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Nucleotide Sequence Analysis of RepFIC, a Basic Replicon Present in IncFI Plasmids P307 and F, and Its Relation to the RepA Replicon of IncFII Plasmids

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RepFIC is a basic replicon of IncFI plasmid P307 which is located within a 3.09-kilobase *Sma*I fragment. The nucleotide sequence of this region has been determined and shown to be homologous with the RepFIIA replicon of IncFII plasmids. The two replicons share three homologous regions, HRI, HRII, and HRIII, which are flanked by two nonhomologous regions, NHRI and NHRII. A comparison of coding regions reveals that the two replicons have several features in common. RepFIC, like RepFIIA, codes for a *repA2* protein with its amino-terminal codons in HRI and its carboxy-terminal codons in NHRI. Although the codons for the *repA1* proteins are located in NHRII, the DNA region containing a putative promoter, ribosomal binding site, and initiation codons is located in HRII. This region also codes for an *inc* RNA. There are nine base-pair differences between the *inc* RNA of RepFIIA and that of RepFIC, and as a result, RepFIC and RepFIIA replicons are compatible. An *Eco*RI fragment from the F plasmid which shows homology with RepFIC of P307 has also been sequenced. This fragment contains only a portion of RepFIC, including the genes for the putative *repA2* protein and *inc* RNA. The region coding for a putative *repA1* protein is interrupted by the transposon Tn*1000* and shows no homology with the *repA1* region of RepFIIA and RepFIC of P307. Our comparative and structural analyses suggest that RepFIC and RepFIIA, although different, have a similar replication mechanism and thus can be assigned to the same replicon family, which we designate the RepFIIA family.

Previously, we described a basic replicon termed RepFIC, present in plasmid P307 and, in a truncated form, in the F plasmid (35). Both plasmids belong to incompatibility group FI (IncFI). We found that RepFIC has homology with a basic replicon of IncFII plasmids called RepA (4), which we refer to as RepFIIA (25). Prior to this finding, plasmids from these two incompatibility groups were believed to be related only in the homology shown by their transfer genes (36). We have shown that in addition to RepFIIA, replicons homologous with RepFIC are widely distributed among plasmids belonging to all six IncF groups (7). In some cases, such as the IncFI plasmids ColV2-K94 and R386, the replicons homologous with RepFIC have been isolated as autonomously replicating miniplasmids (29, 41). In one plasmid, pCG86, we found a chimeric replicon, which is partly homologous with RepFIIA and partly homologous with RepFIC and which was termed RepFIIA/RepFIC (25). On the basis of structural analysis of P307 and pCG86 (24), we concluded that pCG86 was formed by recombination between P307 and an IncFII R plasmid such as R100 or R6 and that a recombination event had occurred between RepFIC and RepFIIA to give rise to the chimeric replicon.

To analyze homologies among replicons homologous with RepFIC in greater detail, we determined the nucleotide sequences of RepFIC isolated from P307 and F. In the present paper, we describe these sequences and compare them with the known sequences of the RepFIIA replicons of IncFII plasmids and the chimeric replicon of pCG86. Our studies indicate that RepFIC and RepFIIA share three highly conserved regions of homology. These observations led us to the conclusion that although the two replicons are compatible, they are regulated in essentially the same manner and are functionally and structurally related. Thus they constitute a family of replicons which we refer to as the RepFIIA family.

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MATERIALS AND METHODS

Bacterial strains. *Escherichia coli* JM101 and JM107 (46) were the host strains for all M13 bacteriophages. *E. coli* C600 (5) was the host strain for plasmids used in this study. PB2960 (PolA$^+$) and PB2961 (PolA$^-$) are prototrophic strains of *E. coli* derived from W3310 and are isogenic except for the *polA1* mutation.

Isolation of single-stranded and double-stranded DNA and purification of DNA fragments. Supercoiled plasmid DNA was isolated as described by Picken et al. (25), and single-stranded bacteriophage DNA was purified as described by Messing (19). Restriction enzymes were purchased from New England BioLabs, and restricted fragments were isolated from low-melting-point agarose gels (SeaPlaque; FMC Corp., Marine Colloids Div.).

Plasmid construction. Plasmid pWM113 was generated by insertion of an *Eco*RI fragment containing RepFIC(P307) into the *Eco*RI site of pBR325 (Fig. 1a). Plasmid pSS3945 (Fig. 1b) was constructed by ligation of the same *Eco*RI fragment to a spectinomycin-streptomycin resistance gene fragment. This fragment, which is called omega, is flanked by transcriptional and translational terminators as well as synthetic polylinkers (*Eco*RI, *Sma*I, *Bam*HI, and *Hin*dIII) (26). To construct pSS3930 (Fig. 1c), the 3.09-kilobase (kb) *Sma*I RepFIC-containing fragment was ligated to the omega fragment.

To construct pSS288 (Fig. 1d), a 288-base-pair (bp) *Sau*3A-*Hin*fI fragment containing the *inc* RNA-coding re-

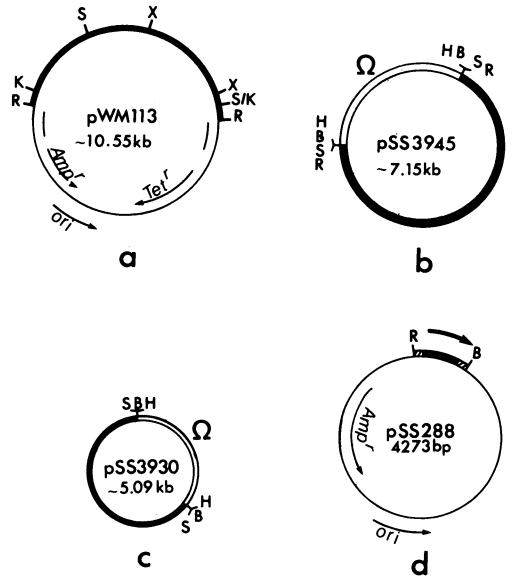


FIG. 1. Schematic representation of some plasmids discussed in the text. For details of the construction of pWM113 (a), pSS3945 (b), and pSS3930 (c), see the text. (a) ▬, Insert; ——, the vector DNA. (b and c), ▭, omega fragment (Ω); ▬, RepFIC DNA. pSS288 (d) was constructed by insertion of the *inc* RNA coding region and its promoter (position 495 to 782; see Fig. 4) into the *Eco*RI-*Bam*HI sites of pBR322 DNA, the latter thus lacking the promoter of the tetracycline resistance gene. The insert does not contain the upstream *repA1* promoter and thus can direct the synthesis of the *inc* RNA only in the direction indicated by the arrow. ▬, *inc* RNA coding region; ▨, RepFIC sequences that flank the *inc* gene; ——, pBR322 DNA. Only some restriction endonuclease sites are marked. Abbreviations: R, *Eco*RI; K, *Kpn*I; X, *Xho*I; H, *Hin*dIII; S, *Sma*I; B, *Bam*HI.

gion of P307 flanked by the *Eco*RI and *Bam*HI polylinkers of M13mp11 was inserted into the *Eco*RI-*Bam*HI sites of pBR322.

The construction of pNZ950 (7), pWM5 (25), and pDXRR3 (44) has been described previously. Plasmid pNZ950 contains the 2.3-kb *Eco*RI fragment f12 of F cloned in pACYC184 (see Fig. 5a).

M13 bacteriophage constructs of RepFIC of P307 and F. The strategy for subcloning of RepFIC(P307) subfragments into M13 bacteriophages is illustrated in Fig. 2. RepFIC was excised from pWM113 (Fig. 1a) by double digestion with *Sma*I-*Xho*I or *Eco*RI-*Xho*I, which yielded three segments, A (*Sma*I-*Xho*I; 1.243 kb), B (*Xho*I-*Xho*I; 1.598 kb), and C (*Xho*I-*Eco*RI; 475 bp). The RepFIC-A segment was digested with *Sau*3A, and the four resultant fragments (60, 102, 331, and 750 bp) were purified. The internal *Sau*3A fragments (60 and 331 bp) were inserted into the *Bam*HI site of M13mp10, and the two terminal *Sma*I-*Sau*3A (102-bp) and *Sau*3A-*Xho*I (750-bp) fragments were inserted into the *Sma*I-*Bam*HI and *Bam*HI-*Sal*I sites of M13mp11 and M13mp10, respectively. The *Sau*3A-*Xho*I fragment was digested with *Hin*fI and *Hae*II, and the blunt-ended fragments were cloned into the *Sma*I site of M13mp11. The left-terminal *Sma*I-*Hin*fI fragment was also cloned in the *Sma*I site of M13mp10. The RepFIC B segment was inserted into the *Sal*I site of M13mp11. The left-terminal *Xho*I-*Pst*I fragment was cloned into the *Sal*I-*Pst*I sites of M13mp11. The 1.598-kb *Xho*I fragment was also digested with *Hin*fI or *Hae*II, and the six resultant fragments were inserted into the *Sma*I site of M13mp11. The RepFIC C segment was inserted into the *Sal*I-*Eco*RI sites of M13mp10 or M13mp11. In some instances, shorter clones were obtained by removal of an internal fragment from a subclone. Occasionally, inserts were turned around to facilitate sequencing from both strands.

The strategy for cloning of RepFIC of F into M13 is shown in Fig. 3. Purified *Eco*RI fragment f12 was sheared, and the sequences of both strands were determined by using shotgun procedures (6). The sequence was also determined from directionally cloned fragments. The *Sma*I-*Bam*HI, *Eco*RI-*Kpn*I, and *Bam*HI-*Eco*RI fragments were cloned in mp18 or mp19, as appropriate. The *Hha*I fragment from bp 346 to 643 (see Fig. 5b) was blunt ended and inserted into the *Sma*I site of mp8. The *Hha*I-*Alu*I fragment from bp 643 to 843 was blunt ended and inserted into the *Sma*I site of mp8. The *Bam*HI-to-*Eco*RI portion (745 bp) of the *Eco*RI fragment f10 (see Fig. 5a) was cloned in both directions in mp8 and mp9, and the sequence of each strand was determined.

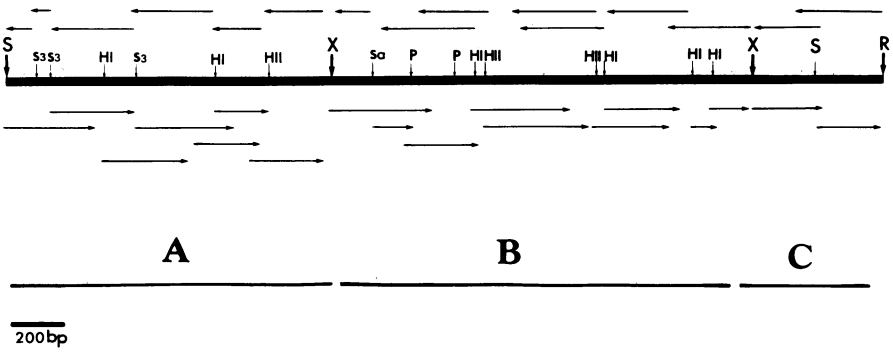


FIG. 2. Sequencing strategy of RepFIC(P307). A (*Sma*I-*Xho*I; 1.24 kb), B (*Xho*I; 1.598 kb), and C (*Xho*I-*Eco*RI; 0.48 kb) are segments of RepFIC. Arrows above the center lines represent sequences derived in the 3′-to-5′ direction; arrows below the center lines represent sequences derived in the 5′-to-3′ direction. The tail of each arrow indicates the position of the insert downstream from an M13 primer hybridization site, and the length represents the approximate sequence read from each clone. The arrowheads do not necessarily indicate the exact position of the last base read, and overlaps have been obtained in almost all cases except the *Xho*I sites dividing A, B, and C. This was not thought to be necessary, because the orientation of the fragments containing the *Xho*I sites had been established by mapping of the adjacent *Sal*I and *Sau*3A sites (for the *Xho*I site dividing the A and B segments) and by mapping of the adjacent *Sma*I and *Pst*I sites (for the *Xho*I site dividing the B and C segments). In addition, the sequences containing the *Xho*I sites were read in both strands, including the overlap of the four bases (5′-TCGA-3′) at the cleavage point. Only relevant restriction enzyme cleavage sites are shown: X, *Xho*; S, *Sma*I; Sa, *Sal*I; P, *Pst*I; R, *Eco*RI; S3, *Sau*3A; HI, *Hin*fI; HII, *Hae*II.

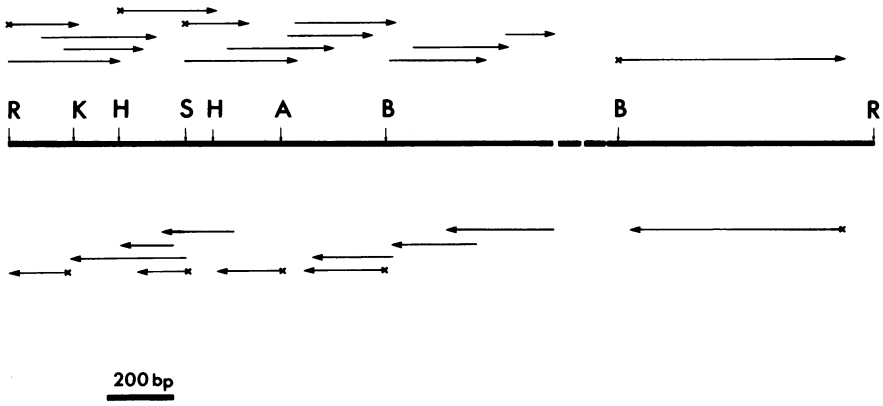


FIG. 3. Sequencing strategy for f12 and f10. The plain arrows indicate randomly cleaved fragments cloned in the *Sma*I site of M13mp8. The tailed arrows indicate directionally cloned fragments. Abbreviations: R, *Eco*RI, K; *Kpn*I, S; *Sma*I, H; *Hha*I, A; *Alu*I; B; *Bam*HI.

RepFIC sequences were determined by the enzymatic chain termination method of Sanger as described by Biggin et al. (8) and Messing (19). Two RepFIC(P307)-specific 17-mer primers, in addition to the M13 17-mer primer (New England BioLabs), were used. These primers were 3'-AAAAGTTCAAGACTTCT-5' (from position 691 through 707) and 3'-CACTAAACCCAAATGCG-5' (from position 902 through 918).

Replication ability of the f12 fragment. The ability of the f12 fragment to support replication of a *polA*-dependent replicon was assayed in two ways. In the first method, competent cells of SC294 (15) were transformed with pNZ950 at 30°C. Purified transformants were grown without selection in liquid medium at 30°C until early logarithmic growth, shifted to 41°C, grown for 15 to 20 generations, and assayed for the number of cells that were resistant to tetracycline (carried on pACYC184) as described by Lane et al. (15). In the second method, the transformation frequency of the isogenic Pol$^+$ and *polA1* strains with pNZ950 was determined at 37°C by estimation of the number of tetracycline-resistant transformants appearing on plates after 2 h of growth under nonselective conditions.

RESULTS AND DISCUSSION

Determination of the nucleotide sequences of RepFIC from P307 and F. The source of RepFIC from P307 was the autonomously replicating miniplasmid pWM101 (25). A 5.15-kb *Eco*RI fragment containing RepFIC was isolated from this plasmid and inserted into the *Eco*RI site in the chloramphenicol resistance gene of pBR325 to generate pWM113 (Fig. 1a). Construction and characterization of different deletion mutants demonstrated that the region required for autonomous replication of RepFIC is located within a 3.09-kb *Sma*I fragment (S. Saadi, Ph.D. thesis, New York University, New York, 1985). This region was ligated to the omega fragment (26) to construct miniplasmid pSS3930 (Fig. 1c). The procedures for obtaining subfragments suitable for sequencing, their cloning into M13 bacteriophages, and the sequencing method used are described in Materials and Methods (Fig. 2).

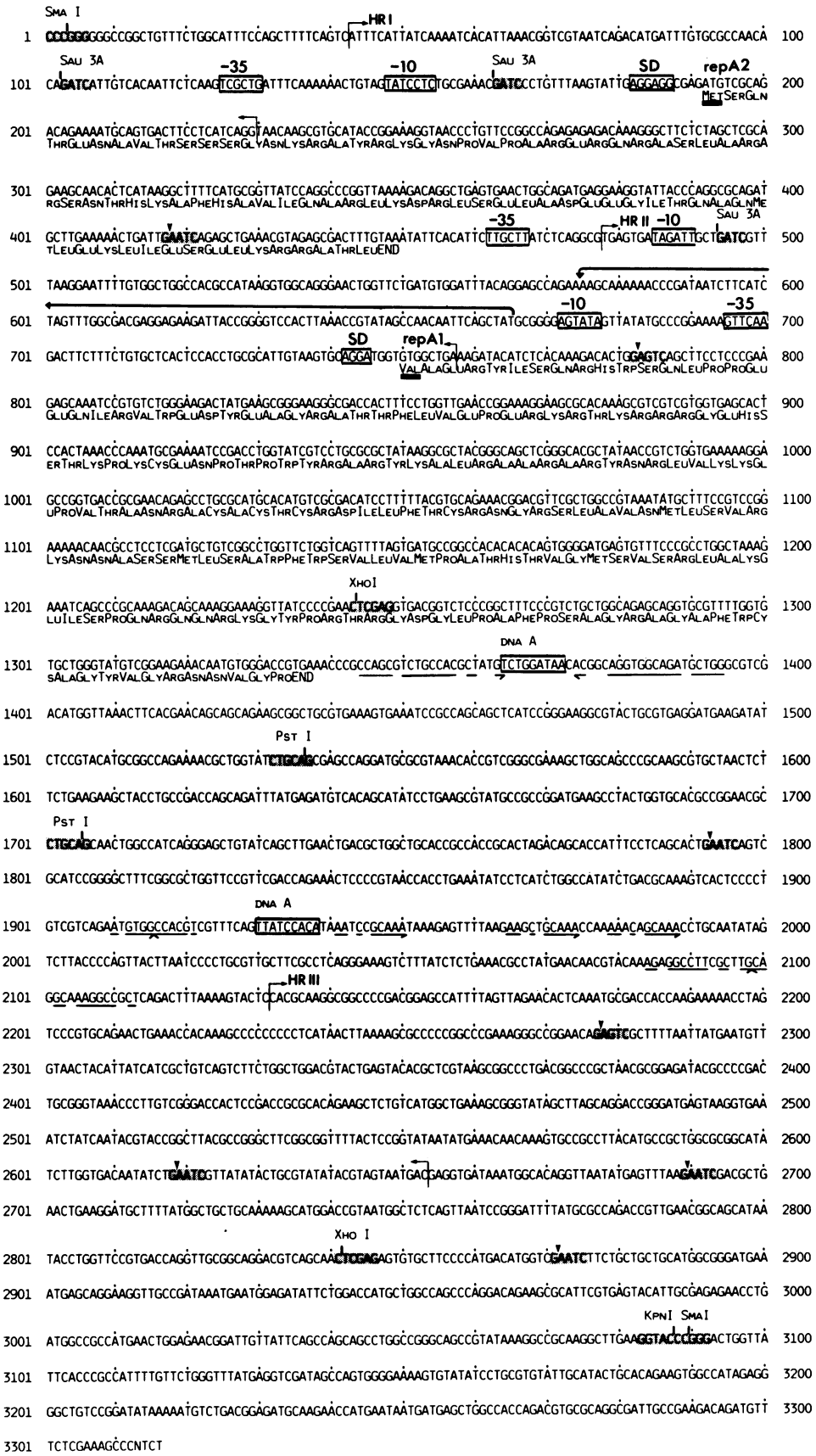
The nucleotide sequence of the 3.09-kb *Sma*I fragment containing RepFIC is shown in Fig. 4. Coordinates are assigned starting with the *Sma*I site in the A fragment, and relevant restriction enzyme sites and putative transcriptional and translational signals are indicated. Significant features of this sequence will be discussed below.

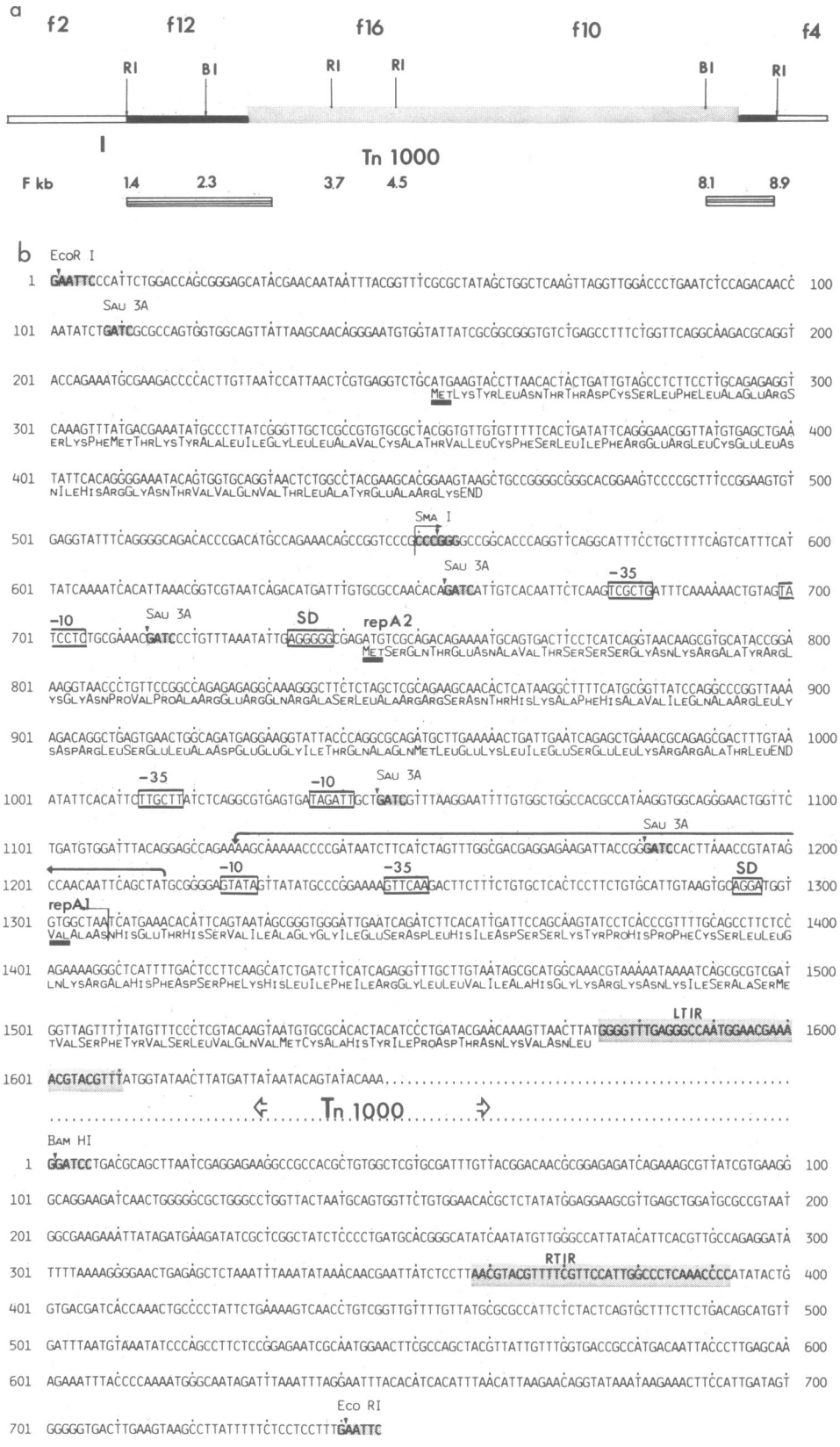
For the sequencing of RepFIC of F, plasmid pNZ950, which contains *Eco*RI fragment f12 of F cloned in pACYC184 (7), was used. The f12 fragment has the coordinates 1.4F to 3.7F and contains the *srnB* gene (2), as well as part of the transposon Tn*1000* (Fig. 5a).

The fragments that were used for sequencing are shown in Fig. 3. The regions of the F plasmid that were sequenced are indicated in Fig. 5a, and the sequences are shown in Fig. 5b. The part of the sequence containing the *srnB* gene (Fig. 5b, positions 252 to 455) has also been determined by Akimoto et al. (3). They reported the sequence from the *Eco*RI site at bp 1 to 654. Except for a base-pair substitution at position 473 and a base-pair insertion at position 468 of f12, their sequence data are in agreement with ours. The significance of the remainder of the sequences shown in Fig. 5b will be discussed below.

Comparison of the RepFIC sequences of P307 and F with the RepFIIA sequences of IncFII plasmids. The complete nucleotide sequences of the RepFIIA replicons from plasmids R1 and R100 (NR1) have been published (30, 33, 34). Except for a 250-bp region of low homology (44%), they have a high degree of homology (96%) over their entire length of 2.7 kb (33). Figure 6 shows a schematic comparison between RepFIC of P307 and RepFIIA of R100. The figure

FIG. 4. The nucleotide sequence of the sense strand of RepFIC of P307. The sequence is numbered starting from the *Sma*I site. The amino acid sequences of the two putative polypeptides discussed in the text, *repA2* and *repA1*, are written below the appropriate coding regions. The −35 and −10 RNA polymerase recognition sites (32) and Shine-Dalgarno sequences (37), which are almost identical to those suggested for R100 (45) and R1, are indicated within the boxes. The first amino acid of each polypeptide is underlined. The arrow above the sequence indicates the location and the direction of *inc* RNA. The location of this RNA was suggested by comparison of the RepFIC and IncFI replicons. The beginning and end of homologous regions (HRI [43 to 230], HRII [378 to 758], and HRIII [2132 to 2654]) are marked. The *dnaA*-binding sites (12) located downstream of *repA1* and within the nonhomologous region II are indicated. Arrows below the sequence indicate the location of direct and inverted repeats. Relevant restriction endonuclease cleavage sites are shown. Arrowheads above the stippled boxes indicate the cleavage site of *Hin*fI.


SMA I
HR1
SAU 3A
-35
-10
SAU 3A
SD
repA2
SAU 3A
HR II
-10
-35
SD
repA1
XHO I
DNA A
PST I
PST I
DNA A
HR III
XHO I
KPN I SMA I


a
f2
f12
f16
f10
f4
RI
BI
RI
RI
BI
RI
I
Tn 1000
F kb
1.4
2.3
3.7
4.5
8.1
8.9
b
EcoR I
Sau 3A
Sma I
Sau 3A
-35
-10
Sau 3A
SD
repA2
-35
Sau 3A
-10
-35
SD
repA1
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RTIR
Tn 1000
Bam HI
Eco RI

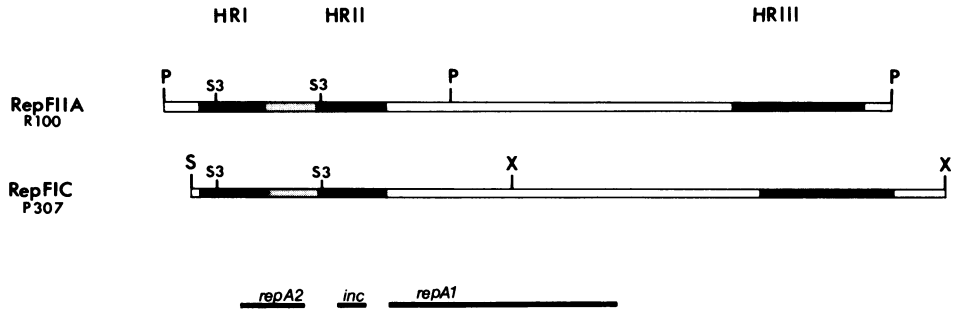

FIG. 6. Schematic presentation of the RepFIC and RepFIIA replication regions. Relevant restriction endonuclease sites and the locations of *repA2*, *inc* RNA and *repA1* of R100 (pSM1) are taken from Rosen et al. (30). The two replicons are aligned for comparison. ▭, Nonhomologous regions; ■, 90 to 100% homology; ▨, about 50% homology. Abbreviations: P, *Pst*I; S, *Sma*I; S3, *Sau*3A; X, *Xho*I.

also shows the 250-bp region of low homology between R100 and R1. It can be seen that there are three regions of high homology, called HRI, HRII, and HRIII, interspersed by two regions of low homology, called NHRI and NHRII. NHRI coincides with the region of low homology between R100 and R1. The existence of these HR and NHR regions had already been deduced from hybridization experiments (Saadi, Ph.D. thesis).

To facilitate a description of the functional organization of RepFIC, it will be helpful to first review the genetic elements of RepFIIA. In the RepFIIA replicon of R100, the genes for mRNAs and their products have been extensively analyzed (31, 45). There are three regulatory genes, two (*repA1* and *repA2*) determining protein products and one (*inc*) determining an RNA product (Fig. 6). There are three promoters, P_C for a polycistronic mRNA for the *repA2* and *repA1* proteins, P_A for a mRNA for the *repA1* protein only, and P_E for *inc* RNA, transcribed in the opposite direction from the *repA1* mRNAs (31, 45). The *repA1* gene product is known to be required for replication initiation, and its level seems to be the key element in replication control (20). The concentration of this protein is determined by *inc* RNA and *repA2* protein, both of which act as negative regulators of replication. The same genes have also been defined in RepFIIA of R1, *repA*, *copB*, and *copA* corresponding to *repA1*, *repA2*, and *inc*, respectively (20). For R1, a 206-bp fragment containing the origin of replication has been delineated (18). For our discussion of the functional elements of RepFIC, we shall use the R100 nomenclature.

The *repA2* gene is located partly in the HRI region and partly in the NHRI region (Fig. 4 [positions 192 to 446] and 6). A comparison of the nucleotide and amino acid sequences for this region from RepFIC of P307 and F and from RepFIIA of R1 and R100 is shown in Fig. 7. The putative promoter region (45), Shine-Dalgarno sequence, and N-terminal end of the structural gene are almost identical for the four replicons. Although they diverge after the amino acid residue 12, there are identical codons and conservative substitutions at many corresponding positions in the NHRI region. There are chain-terminating codons at about the same locations, resulting in the production of four polypeptides with 85, 85, 84, and 86 amino acids for the *repA2* genes of P307, F, R100, and R1, respectively. Although the *repA2* products of RepFIC of P307 and F have not been isolated, it seems most likely that they exist and are similar in function to those of R100 and R1. The sites of action of *repA2* of R100 (11) and *copB* of R1 (16) have been identified genetically and located within the region of the internal *repA1* promoter P_A. The *copB* protein of R1 has been partially purified, and footprinting analysis has defined its binding site within 20 to 25 bp which includes the −35 part of the P_A promoter (28). As the *repA2* protein of R100 and the *copB* protein of R1 are partly different, their targets are expected to be different. This is found to be the case, and as a result, the two *repA2* systems do not cross-react (21). The R1 repressor binding site is different from the corresponding sequence in P307, F, and R100 (Fig. 7, positions 462 to 468).

The *repA1* mRNAs of R100 and R1 which originate at the P_A and P_C promoters have been identified in in vitro experiments (31, 45). The leader transcript portion of the *repA1* mRNA is located entirely within HRII (Fig. 4 and 7). This homology terminates at position 753, after the first codon of the *repA1* protein. After that, the sequences for the *repA1* gene of R1 and R100 are nearly identical, but there is no homology between these and the corresponding sequences of *repA1* for P307 or F. There is also no homology between P307 and F in this region (see below). Thus the presumptive *repA1* protein of RepFIC(P307) is completely different from that of RepFIIA of R100. From the RepFIC sequence (Fig. 4), this protein is calculated to have a size of 22.3 kilodaltons (195 amino acids), whereas the *repA1* proteins of R1 and R100 are 33 kilodaltons.

The *inc* RNA of IncFII plasmids (R100, R6, and R1)

FIG. 5. Restriction map (a) and sequence (b) of the region of F reported here. (a) The position of Tn*1000* in the map of F is based on data in reference 14. ▨, Tn*1000*; ▤, sequenced regions. The coordinates of F are shown in kilobases. f2, f12, f16, f10, and f4 are *Eco*RI fragments 2, 12, 16, 10, and 4 respectively. Abbreviations: RI; *Eco*RI, BI; *Bam*HI. (b) Nucleotide sequence of f12 and a portion of f10. The first part of the sequence is numbered from the *Eco*RI site at 1.4 kb F and extends to position 1645 in Tn*1000*. The left terminal inverted repeat (LTIR) of Tn*1000* commences at position 1574. The complete sequence of Tn*1000* has not been determined here and is not relevant to this communication. Numbering recommences at the internal *Bam*HI site in Tn*1000* and proceeds through the right terminal inverted repeat (RTIR) to the *Eco*RI site at 8.9 kb F. The amino acid sequences of the two putative polypeptides discussed in the text, *repA2* and *repA1*, are written below the appropriate coding regions. The −35 and −10 RNA polymerase recognition sites (32) and Shine-Dalgarno (SD) sequences (37) are indicated within the boxes. The first amino acids for the three polypeptides are underlined. The initiation codon at position 252 is the start of the *srnB* gene (2). The arrow above the sequence at positions 1126 to 1216 indicates the location and transcriptional direction of the putative *inc* RNA. The homology between f12 and RepFIC of P307 begins at position 550 and ends after position 1308 as indicated. Relevant restriction endonuclease cleavage sites are shown.

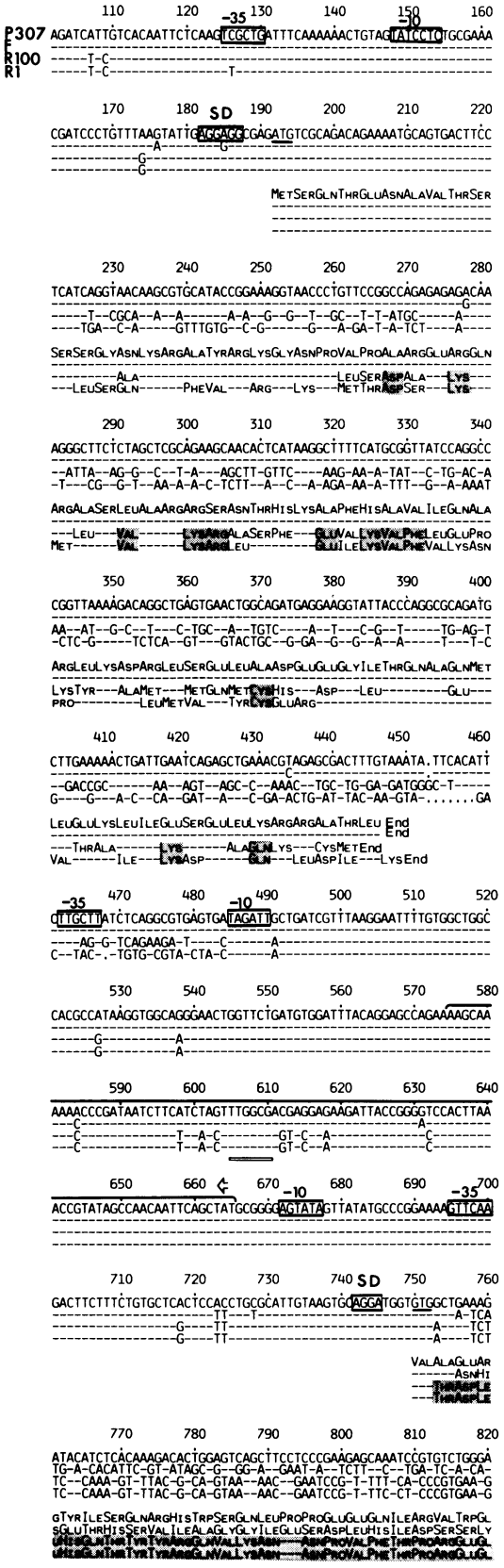

FIG. 7. Comparison of part of HRI and complete NHRI and HRII of RepFIC of P307, F, R100, and R1. Numbers above the upper sequence correspond to RepFIC coordinates. ------, Identical bases as well as amino acids of the four replicons; identical amino acids of R100 and R1. The arrow above the sequence indicates the location and the direction of *inc* RNA. The box below the sequence shows the location of the loop of the major hairpin structure of this RNA.

inhibits replication and determines the incompatibility behavior of these plasmids (16, 39, 44). This RNA, which is identical among R100, R1, and R6, is transcribed within the leader transcript of *repA1*, but in the opposite direction. The target of *inc* RNA is known to be the *repA1* mRNA in the region of complementarity (10, 44, 45).

By analogy to RepFIIA, the sequence of RepFIC shows the existence of a similar RNA at an equivalent position (Fig. 7, bp 575 to 665). It is preceded by a region of reasonable homology to −35 and −10 consensus promoter regions (32). Northern blots and hybridization analyses have demonstrated that a 95-base RNA is transcribed from this region (Saadi, Ph.D. thesis). Furthermore, when this region was cloned into pBR322 (Fig. 1d), the resultant plasmid, pSS288, was able to inhibit the replication of the RepFIC miniplasmid pSS3945 (Fig. 1b; see Table 2). These results provide strong evidence for the existence of an RNA in the RepFIC replicon equivalent to the *inc* RNA encoded by the RepFIIA replicons. There are nine differences in the *inc* RNA region between RepFIC of P307 and the RepFIIA replicon of R100 and R1 (Fig. 7). Significant features of these differences will be discussed below.

The region determining the origin of replication in RepFIIA is less well defined than the genes for the controlling elements. In vitro studies in plasmid R1 have assigned the origin to a 206-bp segment, located 158 bp downstream from the termination codon of the *repA1* gene (18). This finding is in agreement with data on the minimal size of RepFIIA in R100 (22). For RepFIC, we have not localized the origin, but presumably it is located downstream from the *repA1* termination codon and within NHRII (Fig. 4). This presumptive origin region, as well as the region coding for the *repA1* protein, is thus different in RepFIC and RepFIIA. The only similarity we found were two *dnaA* boxes (12) located at positions 1364 to 1372 and 1930 to 1938. A similar *dnaA* box is present in the RepFIIA of R1 and R100 (45) in the same region of the sequence. Binding sites for the *dnaA* protein are present in replicons which require the *dnaA* protein to function, such as the chromosomal origin *oriC* (12) and the origin of phage P1 (1). In this connection, it should be mentioned that elimination of the *Pst*I fragment from plasmid pSS3945 between positions 1536 and 1705 followed by religation abolishes the ability of this plasmid to replicate. Reinsertion of the *Pst*I fragment into the deleted plasmid restores the ability to replicate (S. Saadi, unpublished experiments). The region of the presumptive origin of RepFIC contains a few repeated sequences and dyad symmetries, the significance of which, if any, is not known.

The HRIII segment is located in a part of RepFIIA which, although previously thought to contain the origin of replication (30), subsequently was found to be dispensable for replication of RepFIIA-containing miniplasmids (22). However, it was shown that the removal of this region from pSM1, a miniplasmid derived from R100, results in the formation of polymers (22). Such polymer formation leads to plasmid instability (38). This region may therefore be in-

TABLE 1. Inability of *Eco*RI fragment f12 to support the replication of a PolA-dependent replicon in a PolA$^-$ *E. coli* strain

Plasmid	Replicon	No. of transformants/μg of DNA:		
		PolA$^-$	PolA$^+$	Conclusion[a]
pSS3944	RepFIC	400	575	+
pNZ950	f12/p15A	2	250	−
pACYC184	p15A	20	500	−

[a] + and −, Ability and inability, respectively, to replicate in a PolA *E. coli* strain.

volved in the resolution of polymerized plasmids and may in this way contribute to plasmid stability. The fact that it is also present in RepFIC (positions 2132 to 2654; Fig. 4) suggests that it fulfills a similar function in this replicon. The HRIII region may thus be considered to be an accessory part of the RepFIIA and RepFIC replicons.

A comparison of RepFIC obtained from P307 with RepFIC obtained from F. The homology with RepFIC of P307 starts at position 560 in the nucleotide sequence of the f12 fragment (Fig. 5b) and extends for 749 bp to position 1308. A comparison for most of this region between the two sequences is shown in Fig. 7. As can be seen in the figure, the homology begins 192 bp upstream from codon 1 of the *repA2* gene and extends to codon 2 of the *repA1* gene. There are 10-bp differences between the two sequences (Fig. 7). The region of homology thus contains the complete *repA2* gene and the leader sequence of the *repA1* gene, including the *inc* gene.

The left-hand side of the Tn*1000* insertion is located at position 1575, 265 bp beyond the point at which homology between the RepFIC of P307 and that of F ends. Lack of homology for this 265-bp region with the *repA1* region of either P307 or pSM1 has been confirmed by use of specific probes (7, 35). Insertion of Tn*1000* at position 1575 has presumably inactivated any *repA1*-like gene which may have been present originally. We have examined sequences on the right junction of the Tn*1000* insertion for evidence of homology with the equivalent sequence from RepFIC of P307. Again, no homology was found. This result was confirmed by sequencing the right-hand junction of Tn*1000* with F DNA, by using a 754-bp *Bam*HI-to-*Eco*RI fragment (Fig. 3 and 5a). Results are shown in Fig. 5b. It should be noted that there are no 5-bp direct repeats at the junction of Tn*1000* and F. Such repeats are present in insertion mutants generated by Tn*1000* (27). Therefore, it is likely that deletions occurred at least at one side subsequent to the Tn*1000* insertion in F, which may have deleted part(s) of a *repA1* gene originally present. However, since the HRII region ends at codon 2 in all four replicons (Fig. 5b), it is unlikely that a deletion at the left junction of Tn*1000* with F extended to exactly this point. This supports the notion that a *repA1* protein was originally present in F and that this protein was different from the *repA1* protein of P307.

To confirm that the RepFIC replicon in F is incomplete, we have carried out two kinds of tests for replication in *polA* mutants, as described in Materials and Methods. In the first, the plasmid pNZ950 was transformed into strain SC294, which contains a temperature-sensitive polymerase I. Transformants were selected at the permissive temperature, shifted from 30 to 41°C, and grown for 15 to 20 generations. Subsequently, these transformants no longer grew at 30°C under selective conditions, although they remained viable at 41°C as demonstrated by their ability to grow on nonselective media (data not shown). In the second test, transformations of pNZ950 DNA into an otherwise isogenic PolA$^+$ and PolA$^-$ pair were carried out (Table 1). As controls, DNA from plasmids of similar size with either a *polA*-dependent replicon (pACYC184) or a *polA*-independent replicon (pSS3944) was used. pNZ950, like pACYC184, has a much lower transformation frequency than pSS3944 into the PolA$^-$ strain, whereas the transformation frequencies per microgram of DNA into the PolA$^+$ strain are about the same (Table 1). These results support the conclusion that RepFIC in F is not a functional replicon.

Incompatibility behavior of the RepFIC replicons of P307 and F and the secondary structure of their *inc* RNA. The *inc* gene, whose product is *inc* RNA, determines the incompatibility of IncFII plasmids. The *inc* RNA of R100 not only interacts with its own target but can also recognize the *repA1* leader transcripts of plasmids R1 and R6 and inhibit their replication. This is a general interaction among IncFII plasmids and forms the basis for their incompatibility. RepFIC-*inc* RNAs encoded by pSS288 or pNZ950 or both can inhibit the replication of pSS3945 (Table 2) but do not inhibit the replication of IncFII replicons. Thus IncFII and RepFIC replicons are compatible.

The secondary structure of the *inc* RNA is thought to be critical for its function (for a review, see reference 20). This RNA interacts with its target, the *repA1* leader transcript, and subsequently inhibits translation (17, 43). The structure of the *inc* RNA of R1 has been analyzed (40) by probing with single-strand- and double-strand-specific nucleases. This RNA forms two stem-loop structures; the major one is shown in Fig. 8a. Since base substitutions in or close to the 6-base loop of the major hairpin lead to generation of new incompatibility types (9, 13), it has been suggested that the

TABLE 2. Incompatibility of RepFIC and RepFIIA replicons

Incoming plasmid	Incoming replicon	Resident plasmid	Resident replicon	% Loss of resident plasmid	% Loss in control[a]	Conclusion[b]
pSS3945	FIC(P307)	pWM5	FIIA/FIC	2.5	5	C
pSS3945	FIC(P307)	pRR933	FIIA	2.5	2.5	C
pWM113	FIC/ColE1	pSS3945	FIC	100	17	I
pSS288	FIC(*inc*)/ColEI	pSS3945	FIC	100	17	I
pSS288	FIC(*inc*)/ColE1	pRR933	FIIA	2.5	2.5	C
pNZ950	FIC(*inc*)/p15A	pSS3945	FIC	100	17	I
pNZ950	FIC(*inc*)/p15A	pRR933	FIIA	10	2.5	C
pWM5	FIIA/FIC	pSS3945	FIC	20	17	C
pDXRR3	FIIA(*inc*)/ColE1	pSS3945	FIC	30	17	C
pDXRR3	FIIA(*inc*)/ColE1	pWM5	FIIA/FIC	100	5	I

[a] Control is the stability of the resident plasmid in the absence of selection.
[b] C, Compatible; I, incompatible.

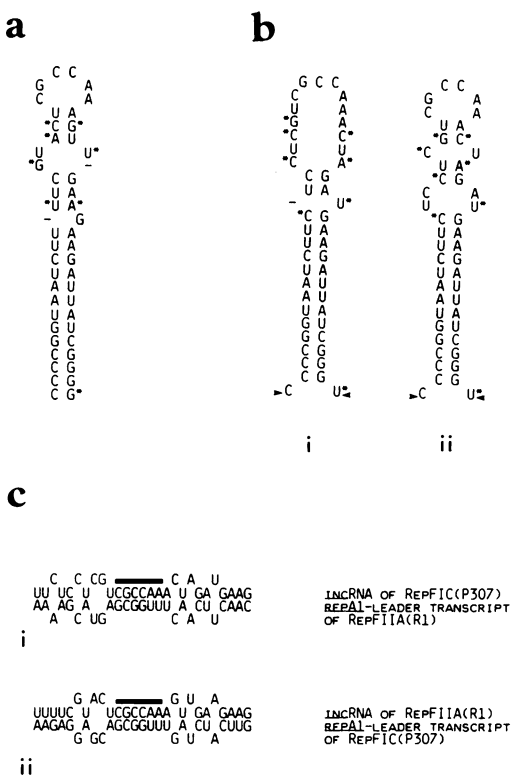

FIG. 8. Folded structure of *inc* RNA. (a) Structure of the major stem-loop of *inc* RNA of R1 (40) (energy = −26.9 kcal). (b) Possible secondary structures of *inc* RNA of RepFIC(P307) predicted by the Zuker and Stiegler algorithm (47). The different bases between R1 and RepFIC(P307) are marked by asterisks. The bases that are different between RepFIC of P307 and F are marked by arows. Structure (i) were predicted by the data of Salser (free energy = −22.6 kcal), and structure (ii) was derived from structure (i) by forcing nucleotides 54 and 55 to base pair with nucleotides 63 and 62 (free energy = −21.7 kcal). (c) Stable base pairing of *inc* RNA of RepFIC with *repA1* mRNA of RepFIIA (i) and of *inc* RNA of RepFIIA with *repA1* mRNA of RepFIC (ii) can possibly be prevented owing to unpaired bases surrounding the conserved loop region (▬).

major site of interaction of the *inc* RNA and its target is this loop.

The above-mentioned structure of the major stem-loop of *inc* RNA of R1 (Fig. 8a) is the same as that determined by the computer algorithm of Zuker and Stiegler (47) with the recommended energies compiled by W. Salser (M. Zuker, personal communication). The *inc* RNA of RepFIC also has the potential to form folded structures. Two possible structures of the major stem-loop of the RepFIC *inc* RNA are shown in Fig. 8b. These structures were predicted by the Zuker algorithm with the data of Salser. Stem-loop (i) is the optimal structure, which has a calculated minimum free energy of −22.6 kcal (1 cal = 4.184 J) and a 15-base loop. This structure is different from that of R1 in Fig. 8a. Williams and Tinoco (42) have shown that structures which have the lowest calculated free energy do not necessarily coincide with those determined from enzymatic cleavage data and may not have biological activity. Although stem-loop (i) corresponds to the lowest free energy, it lacks the 6-base loop. Stem-loop (ii) has an alternate structure, which is derived from stem-loop (i) by forcing nucleotides 54 and 55 to pair with nucleotides 63 and 62. This structure has a calculated free energy of only −21.7 kcal but has the 6-base loop. Thus, although it has a higher energy than the optimal structure (about 0.9 kcal), it may be the functional structure because of the necessity for the 6-base loop.

Our sequence data (Fig. 4 and 7) indicate that there are nine differences between the *inc* RNA of RepFIC(P307) and RepFIIA(R1 or R100 or both) in the bases of the major stem, seven of which are close to the loop. Although the six bases of the loop are conserved (Fig. 7; see the open bar), our incompatibility data (Table 2) show that the RepFIC and RepFIIA replicons are compatible. Base pairing of RepFIC-*inc* RNA with the RepFIIA-leader transcript is destabilized from position 47 to 69 owing to seven mismatches [Fig. 8c, panel (i)]; as a consequence, RepFIC-*inc* RNA has no inhibitory effect on the replication of RepFIIA. For the same reason, RepFIIA-*inc* RNA cannot inhibit the replication of RepFIC.

The *inc* RNA sequence of RepFIC of F possesses two bases that are different from those of P307, both of which are located far from the loop (Fig. 8b). To determine the biological effect of the two base differences, pNZ950 was tested for incompatibility against the miniplasmid pSS3945 derived from P307 and was found to be incompatible (Table 2). Thus, the two base differences have no demonstrable effect on the incompatibility behavior of RepFIC. Like pSS288, pNZ950 does not inhibit replication of pRR933, a miniplasmid derived from R100 (44).

Comparison of the chimeric replicon RepFIIA/RepFIC with RepFIIA and RepFIC. Previously, we had shown that plasmid pCG86 is homologous with plasmid R100 (or R6) for about one-half of its length and with plasmid P307 for the other half (24). One of the switch points in the homology between the plasmids was in a region of homology between the RepFIIA and RepFIC replicons. We isolated an autonomously replicating miniplasmid, pWM5, from pCG86 and determined the nucleotide sequence of the region in which we expected the switches in homology to have occurred (25). At that time, we compared the sequence with the corresponding region of plasmid pSM1, but we had not yet sequenced RepFIC of P307 (25). We now can compare the same sequence with RepFIC of P307 as well as RepFIIA of pSM1 (Fig. 9). This sequence includes most of the HRIII segment and 175 bp to the right of it (Fig. 4 and 6). It can be seen that there are only 2 base-pair differences in the HRIII region between P307 and pCG86, whereas there are about 30 differences between pSM1 and either P307 or pCG86. From these data, it seems that in the formation of pCG86, most of the HRIII region was derived from RepFIC of P307. The remaining 147 bp of pCG86 corresponding to the remainder of the HRIII region have not been sequenced, and we do not know whether there is a switch point in this region in the homology of pCG86 with the two other plasmids. In the 175-bp segment to the right of the HRIII region, there is strong homology between pCG86 and P307 but none between pCG86 and R100.

At the left-hand end of the RepFIIA/RepFIC replicon of pCG86, 323 bp, which includes the leader sequence and most of the codons of the *repA2* gene, has previously been sequenced (23). For this segment, pCG86 is identical with pSM1. We have not sequenced pCG86 beyond this segment to the right; however, we found similarities in the restriction maps between pCG86 and R100, but not between pCG86 and P307, up to the *Hae*II site located in the NHRII region at a point corresponding to location 1850 on the map of RepFIC (Fig. 4). It thus appears that the switch point in homology is somewhere between positions 1850 and 2275 on the RepFIC

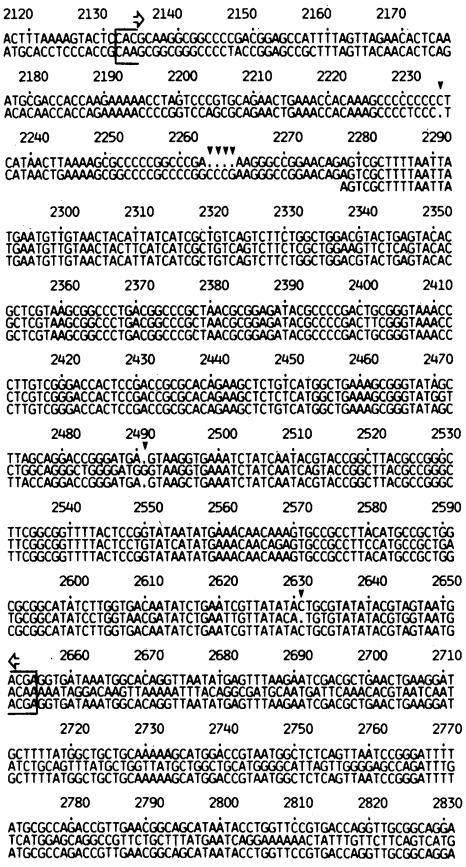

FIG. 9. Comparison of HRIII of P307, pCG86, and R100. Arrowheads indicate insertions and deletions. The beginning and the end of homology between P307 and R100 at bp 2132 and 2656 are marked.

map (Fig. 4) and most probably lies in the HRIII part of this region.

Incompatibility tests are in agreement with the proposed structure of RepFIIA/RepFIC, since pWM5 is compatible with pSS3945 but is incompatible with pDXRR3 (Table 2).

Significance of the homologous regions within the RepFIIA family. As shown above, the RepFIIA family studied here exhibits three regions of homology, HRI, HRII, and HRIII, interspersed by two regions of nonhomology or low homology, NHRI and NHRII (Fig. 4 and 6). NHRI and NHRII contain the genes for the proteins of replication control and their targets. NHRI contains part of the *repA2* protein and its target, the −35 region of the P_A promoter (11). NHRII contains the coding portion of the gene for the *repA1* protein and its target, the origin of replication. The promoters and Shine-Dalgarno sequences of these proteins are located in HRI and HRII. The former contains the promoter P_C for the *repA2* gene, which also functions as a promoter for the *repA1* gene. The latter contains part of the P_A promoter for the *repA1* gene and the gene for the controlling element, *inc*. The role that HRIII plays in replication control, if any, has not been established. Thus, the NHR regions contain the proteins and the HR regions code for the control of these proteins.

Homologous regions favor genetic exchanges which, in the present situation, can lead to the substitution of one replication control protein and its target for another. One can envisage single or double crossovers in the HR regions of RepFIC (Fig. 6) which lead to an alteration in both the *repA2* and the *repA1* proteins and their corresponding targets. For example, a crossover in HRIII and a second crossover outside RepFIC could have led to the formation of pCG86. Similarly, a double crossover in HRI and HRII could have generated the different *repA2* proteins and their targets in R100 and R1. The gene for *inc* RNA lies in a region of homology and may or may not be exchanged when there is a crossover in this region. However, a change of an entire segment of DNA is relatively unimportant, since minor changes in the *inc* gene can bring about a change in the biological behavior of *inc* RNA. As we have shown (Table 2), changes in nine base pairs eliminate the incompatibility between the RepFIC and RepFIIA replicons.

The arrangement seen here, of an overall similar replicon structure with interchangeable parts, thus provides flexibility in the control of replication within the framework of the same basic mechanism. Exchange of replication control elements may be advantageous to a plasmid by altering its incompatibility properties and its copy number. We have shown that replicons belonging to the RepFIIA family are widely distributed among IncF group plasmids (7). It will be of interest to see whether other members of the family exhibit the same basic organization as the replicons studied here. If this turns out to be the case, it will strengthen the argument in favor of evolutionary survival value of a replicon structure composed of a common framework with interchangeable modules.

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