JOURNAL OF BACTERIOLOGY, July 1983, p. 337–344
0021-9193/83/070337-08$02.00/0

Vol. 155, No. 1

Role of Nine Repeating Sequences of the Mini-F Genome for Expression of F-Specific Incompatibility Phenotype and Copy Number Control

HIROSHI TSUTSUI, ASAO FUJIYAMA, TOMOAKI MUROTSU, AND KENICHI MATSUBARA*

Institute for Molecular and Cellular Biology, Osaka University, Kita-ku, Osaka, 530, Japan

Received 1 December 1982/Accepted 20 April 1983

An autonomously replicating 2,248-base-pair DNA segment of the mini-F plasmid carries nine 19-base-pair repeating sequences. Five of the repeats are arranged in one direction and form the right cluster, whereas the remaining four repeats are arranged in the opposite direction and form the left cluster (Murotsu et al., Gene 15:257–271, 1981). Each cluster, cloned separately into the multicopy plasmid vector pBR322, exhibited a strong F-specific incompatibility phenotype (FIP). These clusters were thought to be responsible for the expression of IncB and IncC phenotypes, causing a switchoff function on mini-F replication. Mini-F DNA fragments containing two, three, or more than four repeats were inserted into pBR322. Cells carrying these recombinant plasmids exhibited, respectively, no, intermediate, and strong FIP intensity. Cloning of five repeats into pSC101, whose copy number is about 6 in contrast to 20 for pBR322, resulted in an FIP of intermediate intensity. Thus, the intensity of FIP reflects the dosage of repeats in a cell. The five repeats in the right cluster were eliminated from the mini-F derivative without impairing its autonomous-replicating ability (Bergquist et al., J. Bacteriol. 147:888–899, 1981; Kline and Palchavdhuri, Plasmid 4:281–289). Such deletion, however, caused a sixfold elevation of the copy number. When the eliminated cluster of repeats was reinserted in the derivative, the copy number was lowered to the original value, viz., 1 to 2. The position and orientation of this insertion was not important in the copy number control. Thus, the repeats are also related to copy number control. A model to account for the role of the repeating sequences in the control of copy number and FIP is discussed.

The F plasmid, replicating autonomously in *Escherichia coli* or related bacteria, has a stringent replication control mechanism by which only one or two copies are stably perpetuated per chromosome. Elucidation of the control elements will help us to understand the control mechanism acting in the replication of more complex replicons, such as chromosomes.

It has been shown that in the F genome, all of the replication control elements are located in a limited region, called mini-F (12, 18, 21), which is a 9.1-kilobase DNA segment covering one-tenth of the original F genome. Recently, we have dissected the mini-F region and demonstrated that a 2,248-base-pair (bp) DNA segment can replicate autonomously and that this segment retains the original characteristics for copy number control and expression of F-specific incompatibility phenotype (FIP) (15). This segment has nine nearly identical repeating sequences. Five of the repeats, all arranged in one direction, are clustered (right cluster), whereas the remaining four repeats, all arranged in the opposite direction, form another cluster (left cluster). These two clusters flank a region that can code for a 29-kilodalton (kd) protein with a complete promoter sequence and a ribosome-binding sequence (Fig. 1).

In this paper, we demonstrate that these repeats act in the determination of FIP, as well as in copy number control of the mini-F plasmid.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains used, Km723 and Km1213, are derivatives of *E. coli* K-12 and have been described previously (13). Strain Km723 was used as the host cell carrying recombinant plasmids, except when noted. Strain Km1213 is a subclone of strain JC411 that carries the *polA214*(Ts) mutation (5). The plasmids used are listed in Table 1. All of the media used have been described previously (24). Ampicillin, kanamycin, and tetracycline were used at concentrations of 20, 20, and 10 μg/ml, respectively.

Construction of recombinant plasmids. All of the coordinates in the following description are mini-F coordinates (15). pHT279 and pHT280 are derivatives of pBR322 whose *PstI* site was used to insert an intact

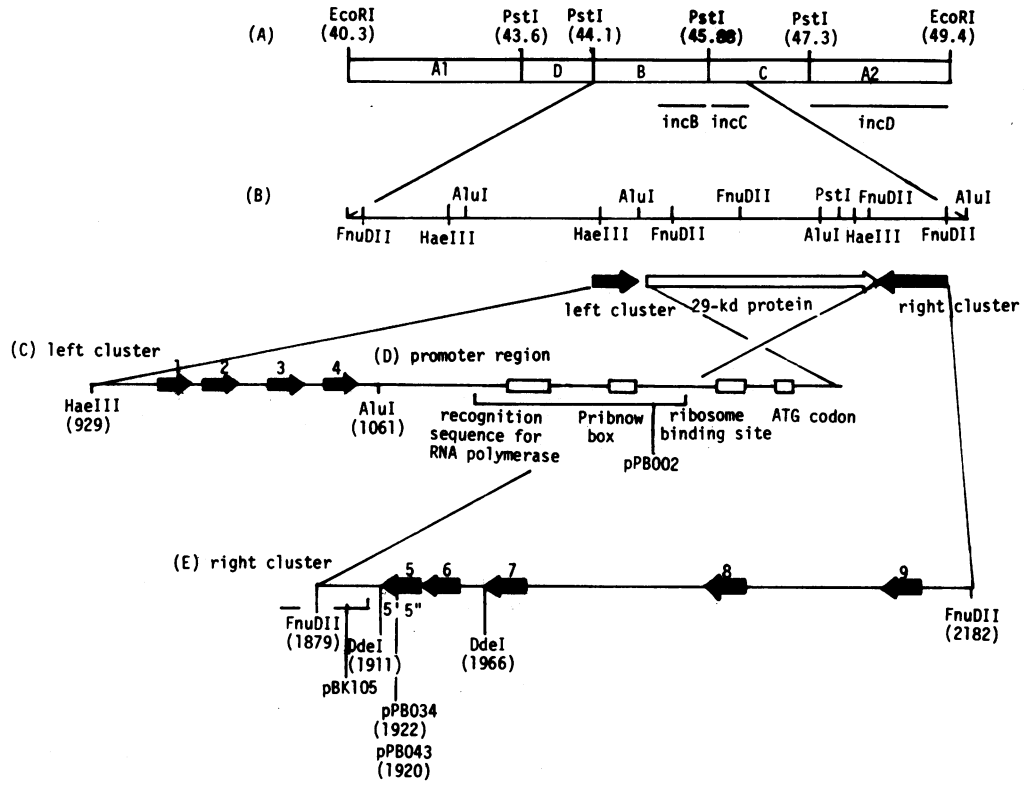

FIG. 1. Genetic and physical structures of mini-F region carrying nine 19-bp repeats. (A) Whole mini-F genome with *Eco*RI and *Pst*I cleavage sites. The names of each segment (A1, D, B, C, and A2) are adopted from Murotsu et al. (15). The regions responsible for IncB, IncC, and IncD are defined by Kline and Lane (7). (B) Segment B and a fraction of segment C, which contribute the minimum replication region defined by Murotsu et al. (15), expanded to show some restriction enzyme cleavage sites and locations of clusters of repeats. The white arrow represents the coding region of the 29-kd polypeptide. (C, D, and E). Structures of the left cluster of repeats, the promoter region for the 29-kd protein, and the right cluster of repeats, respectively. Arrows with numbers represent the 19-bp repeats. Numbers in parentheses represent nucleotide numbers along the mini-F genome as defined by Murotsu et al. (15). pPB002, pBk105, pPB034, and pPB043 represent the sites of Tn*3* insertions (see text). Some of the restriction sites are also shown.

left cluster, viz., a fragment covering 132 bp (*Hae*III coordinate 929 through *Alu*I coordinate 1,061) (Fig. 1 and 2). The insert has been tailed with synthetic *Pst*I linkers. pHT281 and pHT282 are the same as pHT279 and pHT280 except that the inserted fragment is an intact right cluster, viz., a fragment covering *Fnu*DII coordinate 1,879 through *Fnu*DII coordinate 2,182 (Fig. 1 and 2), tailed with *Pst*I linkers. The orientation of insertion in these recombinant plasmids was determined by using *Dde*I. pHT283 and pHT284 carry fragments obtained from *Pst*I-digested pPB034 and pPB043, which are mini-F derivatives carrying Tn*3* within repeat 5 (see Fig. 3). pHT283 carries a fragment covering the site of the Tn*3* insertion coordinate 1,922 through *Pst*I (right end of fragment C, viz., 47.3 kilobase F plasmid coordinate). pHT284 carries a fragment covering *Pst*I (left end of fragment C, viz., 45.88 kilobase F plasmid coordinate) through the site of the Tn*3* insertion coordinate 1,920. These fragments were each inserted in the *Pst*I site of pBR322. pHT309 and pHT311 are derivatives of pBR322, and the *Pst*I site of pBR322 was used to insert, respectively, a 55-bp fragment covering *Dde*I coordinate 1,911 through *Dde*I coordinate 1,966 and a 216-bp fragment covering *Dde*I coordinate 1,966 through *Fnu*DII coordinate 2,182. The cohesive termini of these fragments were filled in by T4 DNA polymerase and tailed with *Pst*I linkers. pHT310 and pHT330 are the same as pHT309 and pHT311 except that the *Pst*I-cleaved vector was replaced by a *Pst*I-cleaved pACYC177. pHT331 is a derivative of pSC101, and the *Eco*RI site of pSC101 was used to insert the same segment cloned in pHT281 and pHT282, except that this segment was further tailed with 18-nucleotide-long *Pst*I-*Eco*RI adaptors (O. Chisaka, personal communication). The cloned segments in these recombinant plasmids are schematically represented in Fig. 2.

Assay for intensity of FIP. The intensity of FIP was assayed as the transduction efficiency with packageable plasmid F (ppF), whose replication is driven by mini-F. It carries an ampicillin resistance gene and is packaged in the λ phage head (10). Bacteria (strain

TABLE 1. Plasmids used

Plasmid	Properties	Source
pBR322	Multiple-copy (copy number, ca. 20) ColE1-type plasmid	H. W. Boyer (2)
pACYC177	Multiple-copy (copy number, ca. 20) ColE1-type plasmid compatible with pBR322	A. C. Y. Chang (3)
pSC101	Multiple-copy (copy number, 6 to 7) plasmid	S. N. Cohen (4)
pML31 (mini-F' *km*)	Mini-F joined to a DNA fragment defining kanamycin resistance phenotype	D. R. Helinski (12)
pPB002, pPB034, pPB043, pBK105	Copy mutants of pML31 whose 40.8–43.1 F coordinate region has been deleted as a result of insertion of Tn*3* followed by deletion (see Fig. 1)	H. E. D. Lane (1), B. C. Kline (8)
pBR322-B25, pBR322-B105	pBR322-based recombinant plasmids carrying fragment B of mini-F (see Fig. 1A) in opposite orientations	T. Murotsu (15)
pBR322-C68, pBR322-C49	pBR322-based recombinant plasmids carrying fragment C of mini-F (see Fig. 1A) in opposite orientations	T. Murotsu (15)
pBR322-A2	pBR322-based recombinant plasmid carrying fragment A2 of mini-F (see Fig. 1A)	N. Komai (9)

Km723) carrying the plasmid(s) to be tested were grown to log phase in PBB medium and infected with ppF at an appropriate multiplicity at 37°C. After 40 min, the mixture was plated on PBB agar containing ampicillin. The number of Amp^r transductants appearing in cells that do not carry the plasmid was taken as 1. Thus, the intensity of FIP is strong when the observed efficiency of Amp^r transduction is low.

Measurement of plasmid copy number. Bacteria (strain Km 1213) carrying a plasmid to be tested were grown at 40°C in PBBYT medium and ampicillin. Cells were labeled with [^{3}H]thymidine and lysed, and the ratio of plasmid DNA in closed circular form was assayed relative to cellular DNA, using CsCl-ethidium bromide density gradient centrifuguation (24).

DNA sequencing. The nucleotide sequence was determined according to the method of Maxam and Gilbert (14).

RESULTS

Direct repeats show FIP. Three segments of mini-F, viz., segments B, C, and A2 in Fig. 1A, show FIP. They are termed *incB*, *incC*, and *incD*, respectively (7). The mechanism of action of *incD* may be different from those of *incB* and *incC*, because *incD* acts on both F and the F-related plasmid R386, whereas *incB* and *incC* act only on F (6).

FIP can be quantitatively assayed by making use of the ability of the resident plasmid to interfere with establishment of the plasmid state by superinfecting ppF. Thus, the decrease in transduction efficiency by ppF represents the strength of the intensity of FIP. Using this assay system, we confirmed that segments B, C, and D exhibited FIP when cloned into pBR322 and that cloned segment B or C showed a much more intense FIP than cloned segment A2. In the studies described below, we concentrate on IncB and IncC expressed by B and C clones. The intensity of FIP expressed by the pBR322-cloned segment B or C was much stronger than that expressed by the autonomously replicating mini-F (Table 2). In those cells that carry pBR322-cloned segment B or C, DNA synthesis of superinfecting ppF was totally switched off. On the other hand, in those cells that carry mini-F, a limited but significant level (10% of control) of ppF DNA synthesis was observed. In cells carrying cloned segment A2, ppF DNA synthesis took place at a rate as high as about 60% of control. The orientation of insertion within the pBR322 genome did not detectably contribute to the intensity of FIP.

The regions of DNA showing IncB and IncC phenotypes are characterized by the presence of 19-bp repeats (Fig. 1C and E). Tolun and Helinski have demonstrated that the right cluster of repeats, when cloned into ColE1, exhibits FIP (22). We confirmed this by showing an intense FIP with a cloned *Fnu*DII fragment (coordinates 1,879 through 2,182) that carries repeats 5 through 9 cloned into pBR322 (Fig. 2c and d). Similarly, the cloned left cluster carrying repeats 1 through 4, extracted by *Hae*III (coordinate 929) and *Alu*I (coordinate 1,061) cleavage, exhibited intense FIP (Fig. 2a and b). Irrespective

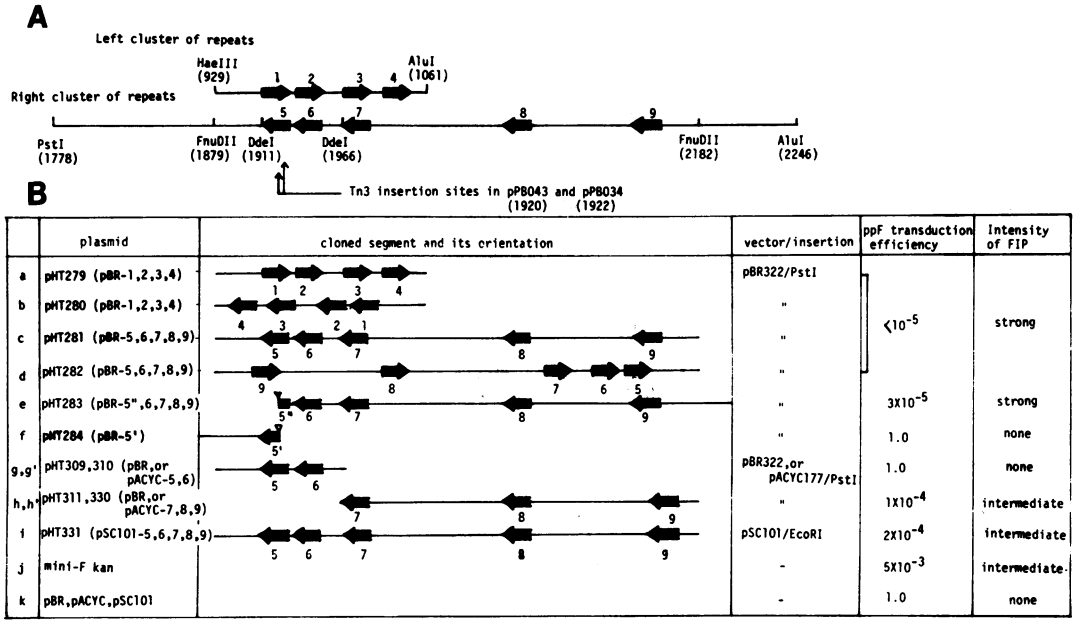

FIG. 2. Cloned fragments and their FIPs. (A) Left and right clusters of repeats (arrows with numbers) to visualize the relative position. Cleavage sites of relevant restriction enzymes and sites of Tn*3* insertions as used in this study are shown. Numbers in parentheses are the nucleotide numbers defined by Murotsu et al. (15) (Fig. 1). (B) Cloned fragments and their orientations in the vector. See the text for details of construction of the recombinant plasmids and measurement of FIP (ppF transduction efficiency). The ppF transduction efficiency varied severalfold in each experiment, but that of pHT283 was always lower than that of intermediate-type plasmids (h, h', i, and j). Low transduction efficiency indicates strong FIP.

of the direction of insertion within the vector, the recombinant plasmids carrying either of the two clusters exhibited intense FIP. No difference in the intensity of FIP was detected whether the recombinants carried the extracted clusters of repeats or the B or C segment. Thus, we conclude that each of the two clusters of 19-bp repeats is responsible for the expression of FIP, viz., IncB and IncC.

TABLE 2. FIP expressed by pBR322-cloned fragment of mini-F or by autonomously replicating mini-F

Plasmid[a]	ppF transduction efficiency[b]	FIP intensity	Switch-off effect on F replication[c]
pBR322-B25	$<1 \times 10^{-5}$	Strong	$<1 \times 10^{-2}$
pBR322-B105	$<1 \times 10^{-5}$	Strong	$<1 \times 10^{-2}$
pBR322-C49	$<1 \times 10^{-5}$	Strong	$<1 \times 10^{-2}$
pBR322-C68	$<1 \times 10^{-5}$	Strong	$<1 \times 10^{-2}$
pBR322-A2	6×10^{-1}	Intermediate	6×10^{-1}
Mini-F' *km*	5×10^{-3}	Intermediate	1×10^{-1}
None	1.0	None	1.0

[a] These plasmids are described in Table 1. The members of the pairs B25-B105 and C68-C49 carry the same insertion with opposite orientations.

[b] Assayed as described in the text (see Fig. 2).

[c] DNA synthesis of infecting ppF was measured by radioisotope labeling with [^{3}H]thymidine followed by sucrose gradient sedimentations as described previously (24). The numbers represent the amount of ppF DNA synthesis in plasmid carrier cells relative to the amount in control, plasmid-free cells.

Dissection of the right cluster of repeats. To dissect the right cluster of repeats into smaller units, we chose two Tn*3* insertion derivatives of mini-F, pPB034 and pPB043 (1). Sequencing results (Fig. 3) have shown that in both of the insertion derivatives, Tn*3* was inserted in repeat 5 in the right cluster. As a result, repeat 5 was separated into 5' and 5" (Fig. 2). Using the *Pst*I cleavage site in Tn*3*, we prepared a fragment carrying repeats 5" and 6 through 9 and cloned it into pBR322. Such a recombinant showed a decreased intensity of FIP as compared with the clones carrying the intact right cluster (Fig. 2e). As expected, a clone carrying only repeat 5', i.e., a half-unit of the 19-bp repeat, failed to show any FIP (Fig. 2f). Restriction endonuclease *Dde*I cleaves the right cluster into two fragments that carry repeats 5 and 6 and repeats 7, 8, and 9, respectively (Fig. 1 and 2). When these two fragments were cloned and tested, two repeats did not exhibit FIP (Fig. 2g), whereas three repeats exhibited FIP of intermediate intensity (Fig. 2h). These results suggest that the

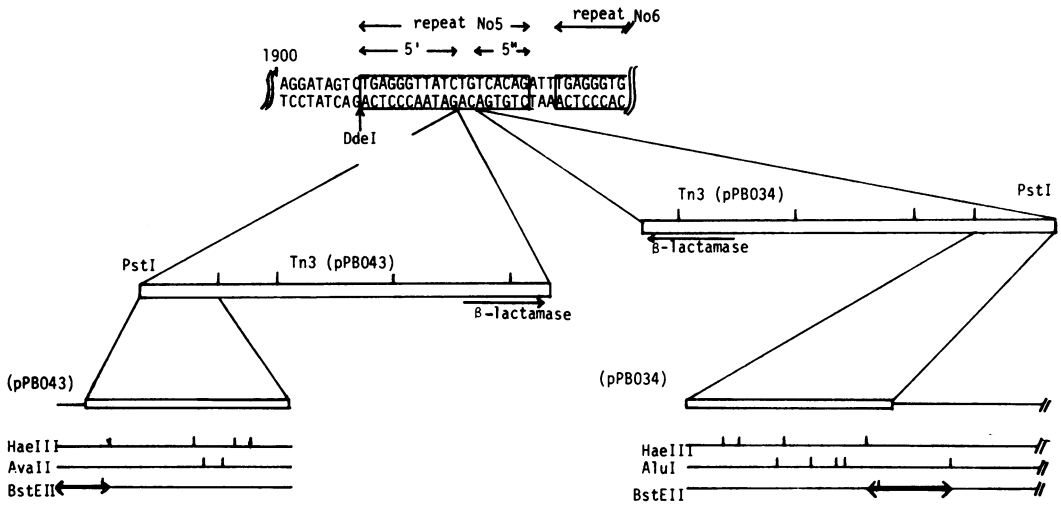

FIG. 3. Nucleotide sequence around repeats 5 and 6 and sites and orientations of Tn*3* insertion in pPB034 and pPB043. DNA from pPB034 and pB043 was cleaved with *Pst*I, and the resulting 2.2-kilobase fragment from pB034 and 1.1-kilobase fragment from pB043 were cloned into pBR322 to form pHT283 and pHT284, respectively. Parts of the cloned fragments were sequenced by the method of Maxam and Gilbert (14). The inserted Tn*3* is schematically shown to represent its orientation. The *Pst*I cleavage sites, which are adopted from Heffron et al. (11), are also shown. At the bottom of the figure the strategy for sequencing is shown. Open boxes indicate the region from Tn*3*.

number of repeats controls the intensity of FIP. However, at this stage we can not eliminate the alternative explanation that in the right cluster, repeats 7 through 9 are primarily responsible for FIP, and repeats 5 and 6 only enhance it.

Number of repeats and intensity of FIP. To examine the relationship between the dosage effect of the repeats and the intensity of FIP, we cloned the intact right cluster of repeats into pSC101. The copy number of this plasmid is 6 to 7 per host chromosome, a value about one-third of that of pBR322 or pACYC177. The resulting recombinant plasmid exhibited FIP of intermediate intensity (Fig. 2i). This observation suggests that the number of repeats carried in a cell determines the intensity of FIP.

To further examine the dosage effect, we constructed a set of bacterial strains each harboring two compatible pairs of plasmids that carry different numbers of repeats. The results in Table 3 demonstrate that the intensity of FIP is additive. This effect can be seen even though the repeats are carried in *trans*.

Direct repeats in the right cluster control the copy number of mini-F in autonomously replicating state. The Tn*3* insertion derivatives we used in this study were originally characterized as copy mutants whose number in a host cell is elevated to 1.5 times the normal number (1). Bergquist et al. and Kline and Palchaudhuri, who used similar insertion mutants, showed that the removal of the region to the right of the

TABLE 3. Intensity of FIP exhibited by pairs of compatible recombinant plasmids each carrying different number of repeats

Plasmids[a]	ppF transduction efficiency[b]	FIP intensity
pHT309 (pBR-5,6) + pHT310 (pACYC-5,6)	1	None
pHT311 (pBR-7,8,9) + pHT310 (pACYC-5,6)	1×10^{-4}	Intermediate
pHT311 (pBR-7,8,9) + pHT330 (pACYC-7,8,9)	2×10^{-5}	Strong
pHT331 (pSC-5,6,7,8,9) + pHT330 (pACYC-7,8,9)	1×10^{-4}	Intermediate
pHT331 (pSC-5,6,7,8,9) + pHT310 (pACYC-5,6)	2×10^{-4}	Intermediate
pHT309 (pBR-5,6)	1*	None
pHT310 (pACYC-5,6)	1*	None
pHT311 (pBR-7,8,9)	1×10^{-4}*	Intermediate
pHT330 (pACYC-7,8,9)	1×10^{-4}*	Intermediate
pHT331 (pSC-5,6,7,8,9)	2×10^{-4}*	Intermediate
None	1.0*	None

[a] Plasmids carrying all or part of the right cluster of repeats are described in Fig. 2. pBR, pACYC, and pSC in parentheses represent vectors. The numbers in the same parentheses represent the repeat. For example, (pBR-5,6) represents a pBR322-based recombinant plasmid that carries repeats 5 and 6 (see Fig. 1 and 2).

[b] Assayed as described in the text. The values with an asterisk are from data in Fig. 2.

insertion sites did not impair replication ability, but the plasmid copy number was elevated (1, 8, 17). We deleted most of Tn*3*, along with segments C and A2 located to the right of it (Fig. 4), from insertion mutant pBK105 (Fig. 1) by cleavage with *Bam*HI followed by recircularization. The resulting plasmid pHT332 (Fig. 4) carries the segment from 43.1 F through 45.99 F (17) along with a 1.4-kilobase DNA fragment of Tn*3* coding for β-lactamase. It does not carry any of the repeats in the right cluster. The copy number of this plasmid was 11 to 13, in contrast to 1 to 2 for an intact mini-F. Apparently the right cluster of repeats is dispensable for a minimum autonomous replication drive, but it acts in keeping the copy number low.

The *Bam*HI ends of the autonomously replicating segment pHT332 were converted into *Eco*RI ends by joining seven-nucleotide *Bam*HI-*Eco*RI adaptors (O. Chisaka, personal communication) and then inserted into the *Eco*RI site of pBR322 that had carried an intact right cluster in the *Pst*I site of the β-lactamase gene (pHT282). As a control, pBR322 carrying a segment of irrelevant DNA of similar size inserted at the same site was also used. In the recombinant pHT545 shown in Fig. 4, the autonomously replicating segment (43.1 F through 45.99 F) and the right cluster of repeats (coordinates 1,879 through 2,182) are in the same orientation as in the original mini-F. In recombinant pHT570, they are in the opposite orientation. In both of the recombinants, the autonomously replicating segment and the right inserted cluster are far separated as compared with the structure in the original mini-F. When the copy number of these plasmids was measured in *polA*(Ts) cells at high temperature, where they replicate by mini-F

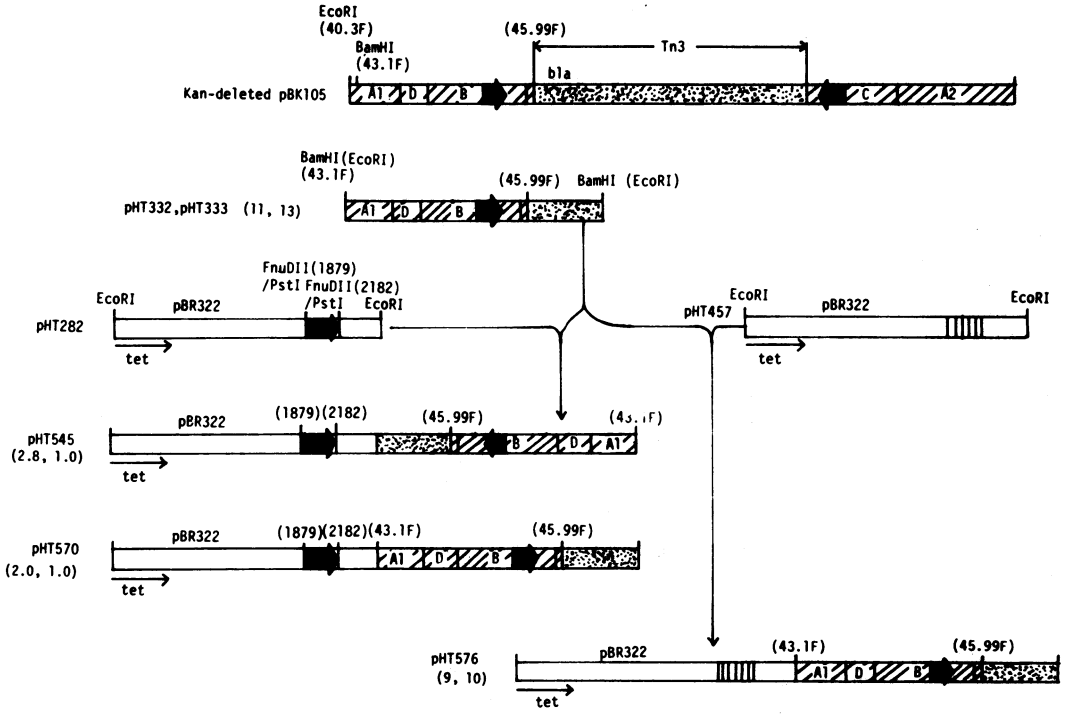

FIG. 4. Genome structures and copy numbers of mini-F derivatives with and without the right cluster. pHT332 was constructed from pBK105 (see Fig.1) by cleavage with *Bam*HI, recircularization, and isolation of an autonomously replicating derivative in a *polA*(Ts) host at nonpermissive temperature (40°C). This plasmid is the same as pBK118 (17). To the *Bam*HI ends of this plasmid were added seven-nucleotide-long *Bam*HI-*Eco*RI linkers to form pHT333. pHT332 and pHT333 replicate by mini-F drive, but do not carry the whole right cluster of repeats or segments C and A2. pHT282 replicates by pBR322 drive, in whose *Pst*I site the right cluster is inserted (see Fig. 2). pHT457 is similar to pHT282 except that a 660-bp DNA segment, from Tn*3* without promoter ( ), substitutes for the cloned right cluster. pHT545 and pHT570 are the cointegrates of pHT333 and pHT282. pHT576 is the cointegrate of pHT333 and pHT457. The plasmids were transformed into a *polA*(Ts) host, and copy numbers were measured under nonpermissive conditions for pBR322 replication (40°C) as described in the text. Each number in parentheses under the name of a plasmid represents the copy number obtained from a different culture. DNA originating from mini-F, Tn*3*, and pBR322 is represented by , , and , respectively. The thick arrows show the right and left clusters of repeats.

drive, pHT545 and pHT570 were found to be kept in low copy number. On the other hand, pHT576 was kept in high copy number. Thus, we conclude that the right cluster controls the maintenance of the low copy number of the mini-F replicon and that its location on the genome, as well as the orientation of the repeats, is not of primary importance. We were not able to tell, however, whether the copy number of pHT545 and pHT570 was exactly the same as that of intact mini-F.

DISCUSSION

An autonomously replicating fragment of mini-F is equipped with three incompatibility loci, *incB*, *incC*, and *incD*. Tolun and Helinski demonstrated that IncC is caused by the right cluster, consisting of five repeats (22). We have confirmed this by cloning a smaller fragment containing repeats 5 through 9. We have further extended the observation to the left cluster, which carries repeats 1 through 4, and have shown that this cluster also exhibited FIP, viz., IncB. The pBR322-cloned cluster of repeats shows an FIP much stronger than that shown by an autonomously replicating mini-F.

The intensity of FIP is correlated with the number of repeats in a cell. Tolun and Helinski reached a similar conclusion by using the right cluster of repeats (22).

A mini-F genome carries nine repeats. As the copy number of this replicon is 1 to 2, 9 to 18 repeats are harbored in a mini-F carrier cell. The FIP of this system was roughly the same as that of pSC101, carrying five repeats. As the copy number of this plasmid is 6 to 7 per chromosome, 30 to 35 repeats must be carried in the cell. It seems that the intensity of FIP is primarily controlled by the number of repeats but at the same time is affected (at least to some extent) by the structure or arrangement of the direct repeats.

The process by which the repeats bring about FIP is not clear at present. Both the left and the right clusters of repeats are unable to code for protein (15). As a likely mechanism of FIP, we propose a "titration model" which assumes that multicopy repeating sequences titrate a positively acting element(s) that is essential for initiation of replication. From our studies described above, the efficiency of titration (T) is determined by a factor (A) inherent to the structure of repeats and the copy number of repeats (N) in a cell. We assume that $T = k \times A \times N$, where k is a constant and T is related to the intensity of FIP or switch-off function. According to this model, replication will be turned off at a high T value because the positively acting element is titrated. On the other hand, replication will be initiated at a low T value. If the positive factor is supplied at a constant level, a replicon carrying the repeats (i.e., the titrator) will establish an autoregulated replication system, maintaining a fixed copy number as a function of N and A. It must be pointed out that the observed intensity of FIP was intermediate with an intact autonomously replicating mini-F. Our previous study (24) demonstrated that the mini-F genome does not replicate as long as its copy number is higher than 2; it replicates only when the copy number is lowered below 2. We can take this as demonstrating that at around two plasmid copies or 18 repeats per chromosome, titration is not effective.

Repeating sequences in DNA interacting with a proteinaceous factor(s) sometimes exhibit cooperativity in binding (16). In addition, naturally occurring repeats are not identical; however, they are closely related. Therefore, experimental values will be different from those of the theoretical titration model. This may be especially important when we use dissected, deleted, or added repeats of natural origin.

Repeating sequences have been discovered in several replicons, such as RK2 (17-bp repeats) (20), R6K (22-bp repeats) (19), λdv (19-bp repeats in the *ori* region) (23), and P1 (18-bp repeats) (S. Austin, personal communication). These repeats, when cloned into high-copy-number vector pBR322, all exhibit interference of replication of the relevant replicon. The repeats in RK2 and λdv have been dissected, and the results showed a correlation between the number of repeats and the intensity of incompatibility (20, 23).

By nucleotide sequence analyses we showed that two insertional copy mutants (pPB034 and pPB043) have insertions in the right cluster of repeats; this is the first direct proof that the repeats in mini-F play a role in its stringent copy number control. An experiment with deleting and reinserting the right cluster of repeats unequivocally demonstrated that the right cluster of repeats controls the copy number of the mini-F replicon. Interestingly, a DNA segment separating the autonomously replicating region and the direct repeats was not important. Neither was it important in the relative orientation of these two elements.

This observation is again in accord with the titration model. In this particular example, loss of the right cluster (titrator) was counterbalanced by a two- to threefold increment of the plasmid copy number, i.e., that of remaining repeats in the left cluster.

The positive factor, of necessity, must be replicon specific. Seekle et al. and Bergquist et al. have isolated another class of copy mutants with mutations that map in the 29-kd protein-coding region of mini-F (1, 17). These observa-

tions can be taken as suggesting that this protein could be the positive factor. Further studies on the role of this 29-kd protein are in progress.

ACKNOWLEDGMENTS

We thank H. E. D. Lane and B. C. Kline for the generous gifts of plasmid strains.

This work was supported by scientific grants from the Ministry of Education, Science and Culture of Japan.

LITERATURE CITED

1. **Bergquist, P. L., R. A. Downard, P. A. Caughey, R. C. Gardner, and H. E. D. Lane.** 1981. Analysis of mini-F plasmid replication by transposition mutagenesis. J. Bacteriol. **147**:888–899.
2. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. L. Betlach, H. L. Heyneker, H. W. Boyer, J. Crosa, and S. Falkow.** 1977. Construction and characteristics of new cloning vehicles. Gene **2**:95–113.
3. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplified multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134**:1141–1156.
4. **Cohen, S. N., and A. C. Y. Chang.** 1973. Recircularization and autonomous replication of a sheared R-factor DNA in *Escherichia coli* transformants. Proc. Natl. Acad. Sci. U.S.A. **70**:1293–1297.
5. **Kingsbury, D. T., and D. R. Helinski.** 1973. Temperature-sensitive mutants for the replication of plasmids in *Escherichia coli*: requirement for deoxyribonucleic acid polymerase I in the replication of the plasmid ColE1. J. Bacteriol. **114**:1116–1124.
6. **Kline, B. C.** 1979. Incompatibility between *Flac*, R386, and F:pSC101 recombinant plasmids: the specifity of F incompatibility. Plasmid **2**:437–445.
7. **Kline, B. C., and H. E. D. Lane.** 1980. A proposed system for nomenclature for incompatibility genes of the *Escherichia coli* sex factor plasmid. Plasmid **4**:231–232.
8. **Kline, B. C., and S. Palchaudhuri.** 1980. Genetic studies of F plasmid maintenance genes. Plasmid **4**:281–291.
9. **Komai, N., T. Nishizawa, Y. Hayakawa, T. Murotsu, and K. Matsubara.** 1982. Detection and mapping of six mini-F-encoded proteins by cloning analysis of dissected mini-F segments. Mol. Gen. Genet. **186**:193–203.
10. **Hayakawa, Y., and K. Matsubara.** 1979. Construction and some properties of packageable plasmid ppF. Mol. Gen. Genet. **169**:107–112.
11. **Heffron, F., B. J. McCarthy, H. Ohtsubo, and E. Ohtsubo.** 1979. DNA sequence analysis of the transposon Tn*3*: three genes and three sites involved in transposition of Tn*3*. Cell **18**:1153–1163.
12. **Lovett, M. N., and D. R. Helinski.** 1976. Method for the isolation of the replication region of a bacterial replicon: construction of a mini-F′*km* plasmid. J. Bacteriol. **127**:982–987.
13. **Matsubara, K.** 1976. Genetic structure and replication of a replicon of plasmid λdv. J. Mol. Biol. **102**:427–439.
14. **Maxam, A., and W. Gilbert.** 1977. A new method for DNA sequence analysis. Proc. Natl. Acad. Sci. U.S.A. **74**:560–564.
15. **Murotsu, T., K. Matsubara, H. Sugisaki, and M. Takanami.** 1981. Nine repeating sequences in a region essential for replication and incompatibility of the mini-F plasmid. Gene **15**:257–271.
16. **Ptashne, M., A. Jeffery, A. D. Johnson, R. Mauer, J. B. Meyer, C. D. Pabo, T. M. Roberts, and R. T. Sauer.** 1980. How the λ repressor and cro work. Cell **19**:1–12.
17. **Seekle, R. W., B. C. Kline, J. D. Trawick, and G. D. Ritts.** 1982. Genetic studies of F plasmid maintance genes involved in copy number control, incompatibility, and partitioning. Plasmid **7**:163–179.
18. **Skurray, R. A., M. S. Guyer, K. Timmis, F. Cabello, S. N. Cohen, N. Davidson, and A. J. Clark.** 1976. Replication region fragments cloned from F*lac*$^+$ are identical to *Eco*RI fragment f5 of F. J. Bacteriol. **127**:1571–1575.
19. **Stalker, D. M., R. Kolter, and D. R. Helinski.** 1979. Nucleotide sequence of an origin of replication of the antibiotic resistance plasmid R6K. Proc. Natl. Acad. Sci. U.S.A. **76**:1150–1154.
20. **Stalker, D. M., C. M. Thomas, and D. R. Helinski.** 1979. Nucleotide sequence of the region of replication of the broad host range plasmid RK2. Mol. Gen. Genet. **181**:8–12.
21. **Timmis, K., F. Cabello, and S. N. Cohen.** 1975. Cloning, isolation and characteristics of replication region of complex plasmid genomes. Proc. Natl. Acad. Sci. U.S.A. **72**:2242–2246.
22. **Tolun, A., and D. R. Helinski.** 1981. Direct repeats of the F plasmid *incC* region express F incompatibility. Cell **24**:687–694.
23. **Tsurimoto, T., and K. Matsubara.** 1981. Interaction of bacteriophage lambda O protein with the lambda origin sequence. ICN-UCLA Symp. Mol. Cell. Biol. **22**:263–275.
24. **Tsutsui, H., and K. Matsubara.** 1981. Replication control and switch-off function as observed with a mini-F factor plasmid. J. Bacteriol. **147**:509–516.