Copy Number Mutations (Cop⁻) of the Plasmid Containing the Replication Origin (oriC) of the Escherichia coli Chromosome: Lethal Effect of the Cop Region Cloned onto a High-Copy-Number Vector on Host Cells

KAZUO YAMAGUCHI AND MITSUYO YAMAGUCHI

Cancer Research Institute, Kanazawa University, Kanazawa 920, Japan

High-copy-number mutants were isolated from an oriC plasmid. They carried insertion mutations within a region (about 470 base pairs) near the uncB gene. When a segment containing this region was cloned onto a high-copy-number plasmid, such a plasmid could be maintained as an intact form only when it was present in a lower copy number.

The replication origin of the Escherichia coli chromosome (oriC) is located between asnA and uncB, around 83 min on the genetic map (6, 12). A 9-kilobase pair (kb) EcoRI segment including oriC has been cloned as a DNA fragment capable of autonomous replication (16). The oriC plasmids containing the EcoRI segment as sole replicator are present in low copy numbers and are rapidly segregated from the cell (3, 8, 13). We describe here copy number mutations (Cop⁻) in an oriC plasmid, pKY16.

The plasmid pKY16 asnA⁺ (Fig. 1) has been constructed by circularization of the EcoRI fragment (15). This plasmid was unstable in E. coli K-12 strain NK1054 asnA asnB recA1 (15). After growth for 10 generations in OC medium (10) with asparagine, 70% of viable cells lost the plasmid. Furthermore, bacterial clones displaying stable Asn⁺ phenotype were frequently detected during growth under selective pressure. These cells may carry the plasmid genome as a state integrated into the host chromosome (5). We isolated two spontaneous mutants, pKY16-1 and pKY16-2, which were more stably inherited. After 10 generations of nonselective growth, 60% of cells retained the mutant plasmid. Copy numbers of these plasmids in cells grown without asparagine were determined by DNA-DNA hybridization experiments (14). To estimate a copy number of pKY16, a fresh clone whose Asn⁺ phenotype was unstable was chosen in every experiment. Table 1 shows that copy numbers of mutants are higher than that of pKY16 even if these numbers are normalized with fractions of cells without plasmid (Asn⁻) or with integrated plasmid (stable Asn⁺) in the populations grown under selective conditions.

Cleavage patterns of pKY16 and pKY16-1 with restriction endonuclease BamHI or HindIII

showed that the BamHI-1 (B₂-B₃ in Fig. 1) or HindIII-3 (H₂-H₃ in Fig. 1) fragment of pKY16 was replaced by a larger fragment, BamHI-1* or HindIII-3* of pKY16-1, respectively (Fig. 2A and B). Digestion of pKY16-1 with PstI produced two additional fragments in place of PstI-1 (P₁-P₂ in Fig. 1) of pKY16 (Fig. 2C). The HaeII digest of pKY16-1 was missing either HaeII-1A or HaeII-1B of pKY16 (Fig. 2D). We concluded that the largest HaeII fragment of pKY16-1 was HaeII-IB because there is no BamHI cleavage site in this fragment (Fig. 2E).
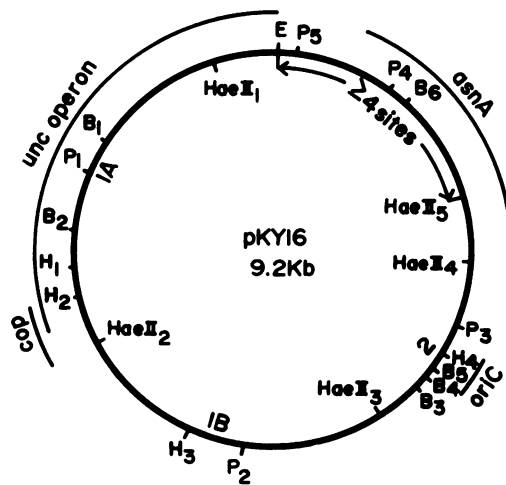

FIG. 1. A restriction endonuclease cleavage map of the oriC plasmid pKY16. The locations of genetic markers and of certain endonuclease cleavage sites (E, EcoRI; B, BamHI; P, PstI; H, HindIII) are indicated. Each cleavage site was numbered counterclockwise. Three HaeII-generated fragments were designated 1A, 1B, and 2 in the order of molecular size.

TABLE 1. Copy numbers of the plasmids[a]

Strain	DNA fixed on filter	Contents[b] (% of whole DNA)	Copy no. of plasmid/host chromosome[c]	Asn+ cells[d] (%)	No. of stable Asn+ cells/total no. of cells[e]
NK1054	HaeII-2 of pKY16	0.053, 0.049			
	HaeII-1B of pKY16	0.056, 0.051			
NK1054 pKY16	HaeII-2	0.178, 0.149	2.5, 2.0	64, 58	1/24, 0/24
	HaeII-1B	0.153, 0.154	1.7, 1.8		
NK1054 pKY16-1	HaeII-2	0.403	7.0	80	0/24
	HaeII-1B	0.358	5.3		
NK1054 pKY16-2	HaeII-2	0.358	6.1	85	0/24
	HaeII-1B	0.512	8.0		

[a] Cells growing in OC medium with (NK1054) or without (others) asparagine were labeled with [³H]thymine at 37°C for three to four generations, and [³H]DNA was isolated as described previously (14). The [³H]DNA (2.4×10^6 to 4.5×10^6 cpm), together with ¹⁴C-labeled HaeII-2 or HaeII-1B fragments of pKY16 (500 to 700 cpm), was incubated with DNA filters in a hybridization mixture (14).

[b] The contents (percent of the whole DNA) of the HaeII-2 segment and the HaeII-1B segment were calculated as follows. From the percentage of [³H]DNA retained on the filter, the value of nonspecific binding to calf thymus DNA ($0.025 \pm 0.015\%$) was subtracted. The result was then divided by the binding efficiency of ¹⁴C-labeled standard DNAs (70 to 80%).

[c] The observed value was corrected for the contribution of a chromosomal HaeII-2 or HaeII-1B segment. Copy number per chromosome was calculated, taking the molecular weight of the E. coli chromosome to be 2.5×10^9, the HaeII-2 segment 1.25×10^6, and the HaeII-1B segment 1.43×10^6.

[d] At the end of labeling with [³H]thymine, cells were spread on a nonselective plate to separate individual cells. Colonies formed were tested for Asn+ by replica plating onto selective medium.

[e] At the end of labeling, cells were spread on a selective plate. Cells from 24 randomly selected colonies were subsequently spread on a nonselective plate to form colonies. The resultant 20 colonies were tested for Asn+. When all 20 colonies derived from a clone were Asn+, the Asn+ phenotype was considered "stable."

Consequently, we conclude that, in pKY16-1, an 0.8-kb segment that has at least one PstI and one HaeII site has been inserted within the HindIII₂-HaeII₂ region (0.47 kb in size). Also, pKY16-2 had a 0.8-kb insert within the same region but at a different site (data not shown). These results suggest that insertion of the DNA segment within the HindIII₂-HaeII₂ region associates with the increase in the copy number of the oriC plasmid. Similar copy number mutants of another oriC plasmid were recently isolated (8). Mutation sites in four insertion mutants were distributed between BamHI₁ and HaeII₂ (10; S. Hiraga, personal communication). None of our present data excludes the possibility that the BamHI₁-HindIII₂ segment also participates in the copy number control of the oriC plasmid.

The HaeII-1A segment, including the HindIII₂-HaeII₂ region, was cloned onto a high-copy-number plasmid, pKY135, that was a derivative of pBR322 and had only two HaeII sites (15). An HaeII digest of a mixture of pKY16 and pKY135 was incubated with T4-DNA ligase (14). Strain NK1037 Hfr dnaA46 recA1 (a derivative of CRT46) was transformed to ampicillin resistance (Amp^r) with the ligated DNA. Strain NK1037 was chosen for a recipient of DNA because of its high efficiency of transformation.

Among Amp^r transformants, cells carrying plasmid pKY159 exhibited slow growth on the plate containing ampicillin because of rapid loss of the plasmid. Analysis of plasmid DNA with restriction enzymes showed that pKY159 was composed of the pKY135 genome and HaeII-1A from pKY16 (Fig. 3A and D). Other plasmids containing both the pKY135 genome and any one of the other HaeII fragments from pKY16 were as stably maintained as pKY135.

When other recA strains, NT101 recA3 (11) and N23-53 recA41 (7), were transformed to Amp^r with pKY159 DNA, transformation efficiencies were two to three orders of magnitude lower than that observed with pKY135. The transformants which resulted were found to carry the normal copy number (20 to 30 copies) of pKY159 derivatives containing insertion or deletion mutations. Figure 3 shows a restriction enzyme analysis of plasmid pKY159 and its insertion mutant, pKY159-16. These results suggest that, in pKY159-16, an approximately 0.8-kb segment containing two additional HaeII sites has been inserted into HaeII-1, BamHI-1, or HindIII-2 of pKY159 (Fig. 3A to C), which have in common the HindIII₂-HaeII₂ segment. This assumption was further confirmed by cleavage with PstI and EcoRI (data not shown).
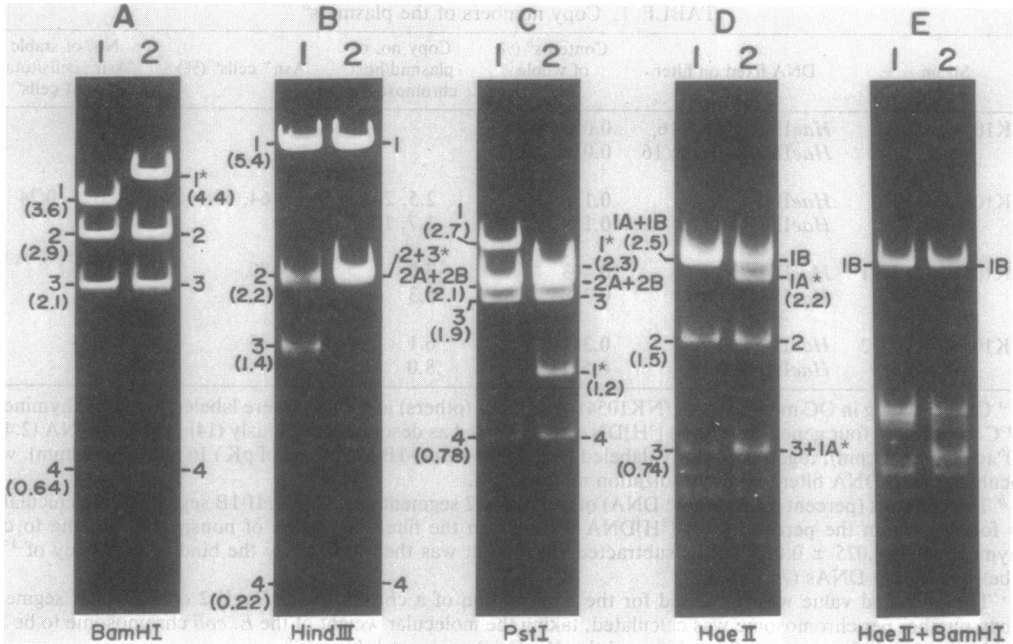
FIG. 2. Agarose gel electrophoresis of restriction enzyme fragments of pKY16 and a copy number mutant, pKY16-1. Plasmid DNA of pKY16 (line 1) or pKY16-1 (line 2) was isolated and digested with BamHI (A), HindIII (B), PstI (C), HaeII (D), or HaeII plus BamHI (E). Agarose gel electrophoresis was performed as described previously (11). Numbers in parentheses indicate molecular sizes of fragments in kb. *, Fragments containing the insert.

We surveyed plasmids from 26 independent transformants of NT101 with pKY159 DNA; of them, 19 were deletion mutants, all of which were missing regions, including the $BamHI_2$-$HaeII_2$ segment. Others were insertion mutants in which a DNA segment 0.8 to 6 kb in size was inserted within the $HindIII_2$-$HaeII_2$ region (five mutants) or the $HindIII_1$-$HindIII_2$ region (two mutants) (data not shown). Inserts in three mutants, including pKY159-16, could be IS1 on the basis of their size (0.8 kb) and cleavage sites of HaeII and PstI (9). Thus, pKY159 could be inherited in NK1037 recA1 as an intact form but not at all in other recA strains tested. We found that pKY135 was present in a lower copy number (about 8 copies per chromosome) in NK1037 cells, compared with 25 copies in NT101 cells. This may be why pKY159 can be maintained in NK1037 cells, since the intact HaeII-1A segment could be kept in low-copy-number plasmids such as oriC plasmids (3, 13) and F (8). It is likely that HaeII-1A has a lethal effect on host cells when the segment is present in about 25 copies per chromosome. The lethality could be released by insertion within the $HindIII_1$-$HaeII_2$ region. A similar lethal effect of a DNA segment near the replication origin of the chromosome cloned onto a high-copy-number plasmid has been recently found in Enterobacter aerogenes and Klebsiella pneumoniae (J. W. Zyskind, N. E. Harding, Y. Takeda, J. M. Cleary, and D. W. Smith, ICN-UCLA Symp. Mol. Cell. Biol., in press).

The $HindIII_2$-$HaeII_2$ region, in which Cop⁻ mutations are located, is included within the $HindIII_1$-$HaeII_2$ segment. Since mutation of the region affected the copy number of the oriC plasmid, it is possible that the lethal effect of the $HindIII_1$-$HaeII_2$ region cloned onto pKY135 is due to its effect on the initiation frequency of chromosome replication. An alternative explanation for the lethal effect of the HaeII-1A segment could be overproduction of Fo components from the unc operon. According to studies on the structure of the unc operon, the uncB, F, and E genes are located on the $EcoRI$-$HindIII_1$ segment (1). Furthermore, recent DNA sequence analysis suggested that HaeII-1A includes the coding region for Fo components (the intrinsic membrane portion of the proton-translocating ATPase) (4). The promoter region of the unc operon has been found in the $HindIII_2$-$HaeII_2$ segment (2). The $HindIII_2$-$HaeII_2$ segment may participate in controlling the expression of the unc operon. It is plausible that overproduction of Fo components affects cell
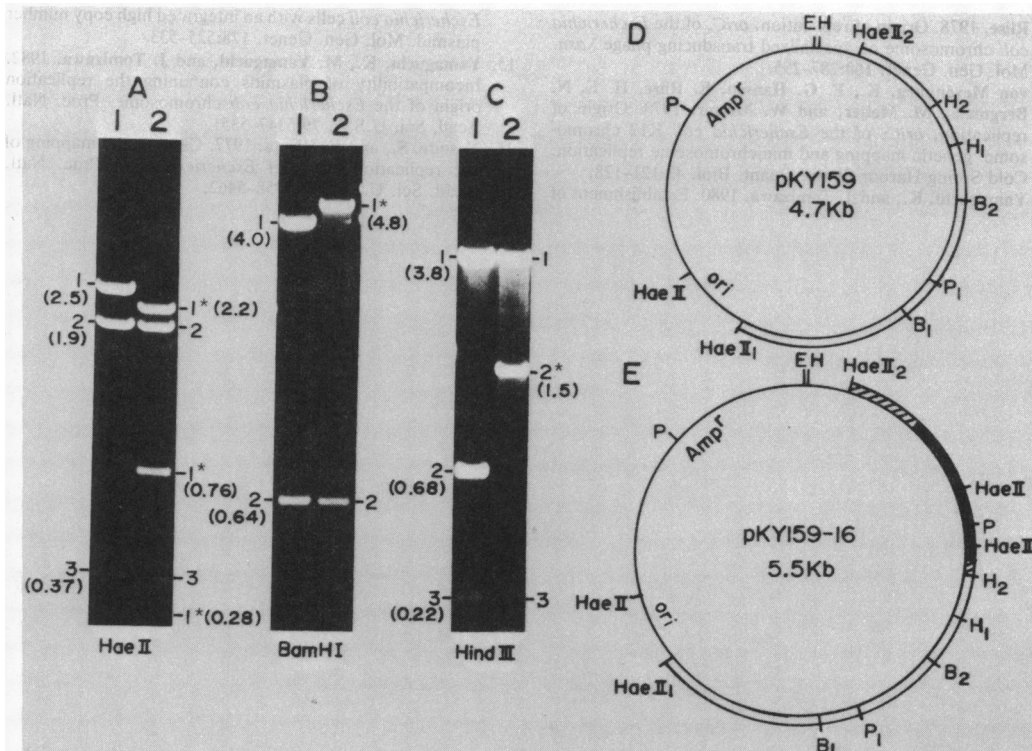
FIG. 3. Agarose gel analysis and cleavage maps of pKY159 and an insertion mutant, pKY159-16. Plasmid DNA of pKY159 (line 1) or pKY159-16 (line 2) was digested with HaeII (A), BamHI (B), or HindIII (C). *, Fragments containing the insert. Certain restriction enzyme cleavage sites (E, EcoRI; B, BamHI, P, PstI; H, HindIII) are shown in (D) for pKY159 and in (E) for pKY159-16. Restriction sites on the segment derived from the E. coli chromosome were numbered as described in the legend to Fig. 1. DNA segments derived from pBR322 are indicated by single lines and those from the E. coli chromosome by double lines. Hatched double lines represent the region within which the insert (heavy line) is located. ori, Replication origin of pBR322.

growth, resulting in the copy number of the oriC plasmid.

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