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A single amino acid alteration in the initiation protein is responsible for the DNA overproduction phenotype of copy number mutants of plasmid R6K

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A novel type of high copy-number (*cop*) mutants of a mini-R6K plasmid were isolated. The mutations were mapped in the *pir* gene which encodes the π initiation protein for plasmid R6K DNA replication. They resulted in an alteration by substitution of a single amino acid: threonine to isoleucine at the 108th position for the *cop41*, and proline to serine at the 113th position for the *cop50*, of the 305 amino acid π protein. The *cop41* mutation in the π protein was found to be *trans*-dominant over the wild-type allele in the copy control of plasmid R6K. Moreover, it was shown that the altered π protein was not overproduced in maxicells carrying this mutant plasmid and had a higher affinity to the repeated sequence which is present in the *pir* promoter region. Most likely the mutated π protein also interacts more efficiently with the same repeated sequences, a target of π, in the replication origin region and increases the frequency of the initiation event per cell division.

Key words: cop mutation/DNA replication/initiation protein/nucleotide sequence/plasmid R6K

Introduction

Bacterial plasmids are model systems for the study of the molecular mechanisms of DNA replication control because of their small size, dispensability for cell viability under certain circumstances, and the fact that plasmid-specific genes determine the rate of initiation of replication resulting in the maintenance of a characteristic copy-number (Nordstrom, 1984; Scott, 1984). We have studied the mechanism of initiation of DNA replication and its control by using an antibiotic resistance plasmid R6K. The plasmid R6K exhibits characteristic properties: it is 38 kilobase pairs (kb) in size and has a copy-number of 15–20 per chromosome. This plasmid encodes ampicillin and streptomycin resistance and is conjugative (Kontomichalow *et al.*, 1970). A 4-kb segment of R6K contains three replication origins (designated *oriα*, β and γ) which are active *in vivo* (Crosa, 1980) and *in vitro* (Inuzuka *et al.*, 1980; Inuzuka, 1985). In addition, this region contains a structural gene, *pir*, for π protein which is absolutely required for the initiation of replication at the three origins *in vitro* (Inuzuka and Helinski, 1978b; Inuzuka, 1985) and *in vivo* (Kolter *et al.*, 1978). The *pir* gene is located between the *oriβ* and γ, and its nucleotide sequence is known (Kolter *et al.*, 1978; Stalker *et al.*, 1982; Germino and Bastia, 1982). Moreover, the π protein binds to the seven directly repeated sequences within the *oriγ* region for the activation of replication at each of the three origins and the 8th repeated sequence near a promoter of the *pir* gene for the autoregulation of its expression (Inuzuka *et al.*, 1982; Germino and Bastia, 1983; Shafferman *et al.*, 1982; Filutowicz *et al.*, 1985; Kelley and Bastia, 1985).

We have found that a temperature-sensitive (*ts*) replication mutant of a mini-R6K derivative produces a thermo-labile π protein and has a reduced copy-number at a permissive temperature (Inuzuka and Helinski, 1978b; Kolter *et al.*, 1978). Furthermore, we also know that partial deletions of the π binding site within the *oriγ* region result in a reduction of binding efficiency of the π protein and a decrease of the copy-number (Inuzuka *et al.*, 1982; Kolter and Helinski, 1982; McEachern *et al.*, 1985). These results suggest that the copy-number of R6K is influenced by the structural alterations of the π protein and its target DNA, probably due to their changed interaction. Therefore, we hoped to find a mutant plasmid that had an altered copy-number by mutations of the *pir* gene or the π target DNA. We report here the isolation and characterization of two high copy-number (*cop*) mutants of a mini-R6K. These mutations map in the *pir* gene and result in amino acid substitutions in the π protein. The *cop* mutations are *trans*-dominant over the wild-type concerning the copy number control. The properties of the mutations suggest that, rather than being overproduced, this mutated π protein has an increased affinity for the repeated sequence DNA which is present in the *oriγ* and *pir* promoter regions. The relationship between the structure and function of this initiation protein will be discussed.

Results

Isolation and some properties of high copy-number mutants of pGN7

Plasmid pGN7 is a 3.96-kb derivative of R6K containing the *oriγ* and *pir* genes and a β-lactamase gene, *bla* (Figure 1). The activity of the β-lactamase is dependent on the gene dosage, namely, proportional to plasmid copy-number, and it has been used to select mutant plasmids with increased copy-numbers (Uhlin and Nordstrom, 1977). Furthermore, a novel procedure was developed to isolate *cop* mutants from a plasmid of relaxed-type replication using plates containing elevated levels of ampicillin and the related antibiotic, methicillin (Wong *et al.*, 1982).

pGN7 DNA treated *in vitro* with hydroxylamine was transformed into YS1 cells. The cells were plated onto L agar containing 6 mg/ml of ampicillin and 4 mg/ml of methicillin (A_6M_4). Under these conditions, YS1 cells carrying the wild-type pGN7 cannot form colonies. Five mutants were independently obtained that expressed a high level of resistance to ampicillin and methicillin and an increased copy-number (Figure 2). These mutants produced a higher amount of β-lactamase. Two of these mutants, designated pGN7*cop41* and *cop50*, have copy-numbers of ~100 per chromosome, whereas the copy-number of pGN7 was ~20. The genetic and biochemical properties of the two *cop* mutants were studied.

First, the incompatibility of pGN*cop41* and *cop50* mutants towards a mini-R6K derivative, pRK419, was tested. These *cop* mutant DNAs were transformed into MV12 cells carrying pRK419, and subsequent loss of the resident plasmid was observed at almost the same frequencies as that by pGN7. This result

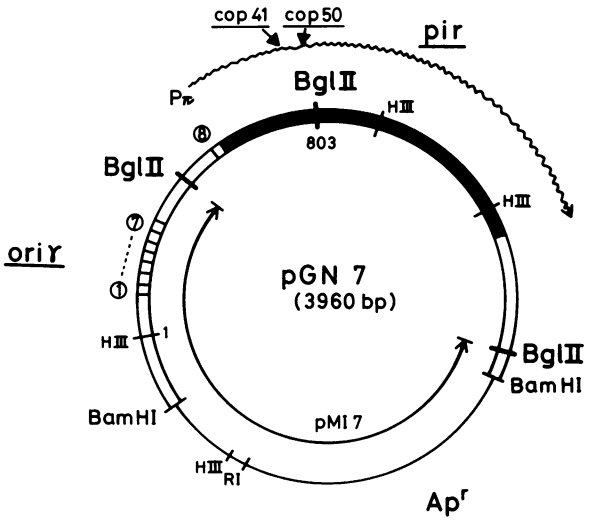

Fig. 1. Physical and genetic maps of pGN7 and its derivatives. The fragment derived from R6K is shown with a double line. *Oriγ* refers to the replication origin which contains the seven direct repeated sequences shown with small boxes (1−7). *Pir* indicates the structural gene for the π protein. The 8th repeat sequence exists in the promoter region (Pπ) of the *pir* gene. The sites of *cop* mutations are marked with arrows within the *pir* gene. HIII refers to a *Hind*III site. *Bgl*II digestion of the pGN7 DNA gives three fragments (2374, 1069 and 527 bp). To construct an *oriγ* plasmid, pMI7, the *pir* gene was deleted by the digestion with *Bgl*II.

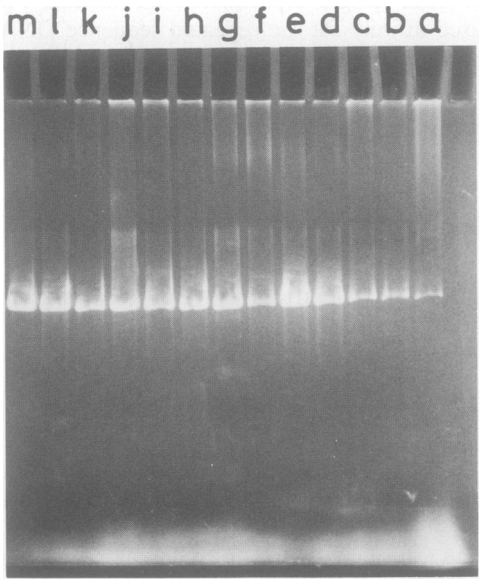

Fig. 2. Agarose gel electrophoresis of the *cop* mutant DNA. The DNA was prepared from high ampicillin-resistant clones by the cleared lysate method. A constant amount of nucleic acids measured by absorption at 260 nm was digested with *Eco*RI to make linear molecules and run on 1.2% agarose gels. **Lane a**, pGN7; **lane e**, pGN7*cop50*; **lane g**, pGN7*cop41*; the other lanes were other clones which were not characterized in this work.

indicates that the *cop* mutants still express strong incompatibility and suggests that the *cop* mutations are not associated with the incompatibility function of the R6K replication.

Mapping of the cop mutations

To confirm that the *cop* mutations were located on their plasmid genome, *cop* mutant DNA was introduced into another strain which then also expressed a high level of ampicillin-resistance.

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          105          108                 113
      -Leu-Pro-Phe-Thr-Ala-Lys-Asn-Ser-Pro-Glu-Gly-Leu-Asp-Leu-
wild  --CTG CCC TTT ACT GCT AAA AAC TCC CCT GAA GAG TTA GAT CTT --
      --GAC GGG AAA TGA CGA TTT TTG AGG GGA CTT CTC AAT CTA GAA --
                                                  BglII
                  -Ile-
cop41 --CTG CCC TTT ATT GCT AAA AAC TCC CCT GAA GAG TTA GAT CTT --
      --GAC GGG AAA TAA CGA TTT TTG AGG GGA CTT CTC AAT CTA GAA --
                    ↑
                   778
                                      -Ser-
cop50 --CTG CCC TTT ACT GCT AAA AAC TCC TCT GAA GAG TTA GAT CTT --
      --GAC GGG AAA TGA CGA TTT TTG AGG AGA CTT ATC AAT CTA GAA --
                                      ↑
                                     792
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Fig. 3. Nucleotide sequences of the *pir* gene containing the *cop* mutations. Only the relevant regions close to the mutation sites are presented. The numbers above the amino acids refer to the altered amino acids in the 305 amino acid π protein. Numbers with arrows indicate substituted bases. Nucleotide 1 corresponds to the *Hind*III-cleavage site in the *oriγ* region.

At least two factors on the R6K genome, an initiation protein π and its target (the *oriγ* region), may contribute to the copy control. To examine whether the *cop* mutations are located in the *oriγ* region, the *cop41* or *cop50* DNA was digested with *Bgl*II and ligated to construct an *oriγ* plasmid, pMI7, deleting the *pir* gene (Figure 1). The resultant plasmids complemented in *trans* with the π protein in φ 1(λ-*pir*$^+$) cells could not grow on a A_6M_4 plate and the copy-numbers of these *oriγ* plasmids were as low as that of pGN7. Moreover, we knew that the π protein acts equally in replication in either a *cis* or *trans* configuration to the origin, because the single cell resistances to ampicillin of pGN7*cop41* and pMI7 in the presence of a *pir41* gene in *trans* were the same. These results mean that the *cop* mutations are not located within the *oriγ* region.

We then carried out marker rescue experiments. pGN7 DNA gives three *Bgl*II fragments of 2374, 1069 and 527 bp, as shown in Figure 1. Three fragments of the *cop41* and *cop50* DNA were examined to see which had the ability to express the cop phenotype by replacing the corresponding pGN7 fragments. The results were that only recombinant plasmids consisting of the smallest fragment derived from the *cop41* or *cop50* DNA had high ampicillin resistance, regardless of the origin of the other fragments. The copy-numbers of the recombinants were analyzed; the high ampicillin resistance clones exhibited 5-fold increased copy-numbers in comparison with that of pGN7. These results show that the mutations responsible for the increased copy-number in the *cop41* and *cop50* mutants are mapped to the 527-bp fragment that includes a part of the *pir* gene.

Identification of the cop41 and cop50 mutations in the pir gene

The nucleotide sequences of the smallest *Bgl*II fragments were then determined and compared with those of pGN7. In the *cop41* mutant, a single base substitution from C to T was found at position 778 within the *pir* gene. This change was located in the second position of the 108th codon of the *pir* gene and resulted in a single amino acid alteration from threonine (ACT) to isoleucine (ATT) (Figure 3). Similarly, in the *cop50* mutation C was changed to T at nucleotide 792 and this base change caused an amino acid change wherein proline (CCT), the 113th amino acid residue of the π protein, was substituted with serine (TCT). Both mutations are a C to T transition and are consistent with the known mode of action of hydroxylamine, which interacts only with C residues. No other change was found in the remainder of the *pir* gene which included the operator region. Therefore, we must conclude that single amino acid substitutions in the π protein are responsible for the DNA overproduction phenotype in the *cop41* and *cop50* mutants.

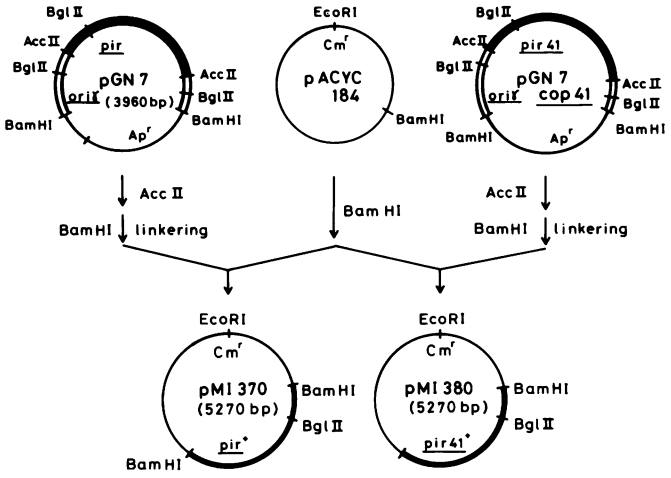


Fig. 4. Cloning of the *pir* and *pir41* genes onto pACYC184. *Pir* and *pir41* refer to the structural gene for the π protein in the wild-type and *cop41* mutant, respectively. The *pir* (*pir41*) gene was isolated as an *Acc*II fragment of 1270 bp (shown with thick lines) followed by *Bam*HI linkering and was ligated into the *Bam*HI site of pACYC184. The recombinant plasmids were selected as clones which are able to support the replication of pMI7.

Table I. Effect of co-existing *pir* plasmids on the copy-number of R6K plasmids

R6K plasmid	*Pir* plasmid[a]	Sensitivity to A_6M_4[b]	Copy number
pGN7	–	S	Low
pGN7	pMI370	S	Low
pGN7	pMI380	R	High
pGN7*cop41*	–	R	High
pGN7*cop41*	pMI370	R	High
pGN7*cop41*	pMI380	R	High
pGN7*cop50*	–	R	High
pGN7*cop50*	pMI370	R	High
pGN7*cop50*	pMI380	R	(High)
pMI7	pMI370	S	Low
pMI7	pMI380	R	High

[a]pMI370: pACYC184-*pir*$^+$, pMI380: pACYC184-*pir41*$^+$.

[b]A_6M_4: ampicillin 6 mg/ml and methicillin 4 mg/ml in L-agar plates.

Cop41 mutation is dominant over the wild-type of the π protein

To examine whether a *cop* mutated π protein is dominant or recessive to the wild-type π protein regarding the control of plasmid copy-number, the wild-type (*pir*$^+$) and *cop41*-mutated *pir* (*pir41*$^+$) sequences were first cloned onto a vector pACYC184 which is compatible with a R6K plasmid. Resultant plasmids, pMI370 and pMI380, carry the *pir*$^+$ and *pir41*$^+$ genes, respectively, as shown in Figure 4. Then, pMI370 or pMI380 was introduced into MV12 cells carrying pGN7 or its *cop* mutants, and the copy-numbers of the R6K replicon plasmids and resistance levels to ampicillin were analyzed. The results are presented in Table I. When the wild-type π protein expressed by pMI370 was supplied in *trans*, all pGN7, pGN7*cop41* and *cop50* maintained their own characteristic levels of resistance to ampicillin and their own copy-numbers. On the other hand, when pMI380, which produces the *cop*-mutated π protein, co-existed in cells harboring pGN7, the copy-numbers of pGN7 increased ~5-fold, like that of pGN7*cop41*, and the cells became resistant to high concentrations of ampicillin and methicillin. The *oriγ* plasmid of 2.4 kb, pMI7, also exhibited an increased copy-number when the altered π protein was supplied in *trans* by pMI380 (Table I). These findings indicate that the mutation in the π structural gene is responsible for the cop phenotype. Moreover, the *cop41* mutation is dominant over the wild-type specified by pGN7, and results in the overinitiation of the co-existing pGN7 or pMI7. In addition, it is also suggested that the *cop* mutation is not the result of the inactivation of a repressor-like factor. No critical effect of the functionally altered π protein was observed toward the pGN7*cop41* plasmid.

Biosynthesis of the π protein in the cop mutants

The expression of the *pir* gene is autoregulated by the interaction of the π protein and the promoter region containing a repeated sequence. Why do the *cop* mutations in the *pir* gene cause an increase of the copy-number? Do structural alterations of the π protein release the autoregulation and result in the overproduction of the initiation protein?

The amount of plasmid-encoded proteins synthesized in maxicells were analyzed and compared using the wild-type and mutants. Plasmid pGN7 and its *cop* mutants were introduced into KE721 (*recA uvrA*) cells, and ^{3}H-labeled polypeptides synthesized in maxicells were analyzed on polyacrylamide gels, as described in Materials and methods. These plasmids specified two polypeptides, π protein and β-lactamase, as shown in Figure 5(d, e and f). A polypeptide of β-lactamase was synthesized in the *cop41* and *cop50* mutants in the range of 2- to 3-fold relative to pGN7. This difference is consistent with the dosage of the *bla* gene. Therefore this procedure can be used to compare the relative amount of plasmid-encoded proteins. In contrast to this, the π protein, a 35-kd polypeptide, was produced in almost the same amount by pGN7 and mutant plasmids. These results lead us to suggest that mutated π proteins autoregulate the expression of the *pir* gene. Moreover, the information obtained from the quantitative comparison of the π proteins which are encoded by pMI370 and pMI380 clearly indicates that it is unlikely that the mutated π protein is overproduced in the *cop* mutant (Figure 5). The ratio of the π protein to chloramphenicol transacetylase in pMI380 carrying the *pir41*$^+$ gene was less than that in pMI370. This observation may indicate that the mutated π protein represses the *pir* expression more stringently than the wild-type because the copy-number of pMI370 and pMI380 is the same.

Increased affinity of the cop-π protein to the repeated sequence

A second possible explanation concerning the *cop* mutation is that a structural alteration of the π protein in the *cop* mutant may enhance the affinity of its binding to the *ori* DNA and/or host initiation proteins which results in the increased copy-number. The π protein binds to the seven direct repeats in the *oriγ* region and 8th repeat in the *pir* promoter, as described earlier. Therefore, a translational fusion between the *pir* gene and the *lacZ* gene was constructed using the *lac* fusion vector pMC1403 (Casadaban *et al.*, 1980). An *Eco*RV-*Alu*I fragment (380 bp) was prepared from pMI372 and contained the *pir* promoter including the 8th repeat and the following coding sequence of 20 amino acid residues of the π protein. This fragment was then ligated to the *Sma*I site in pMC1403 creating an in-frame translation fusion between the *pir* and *lacZ* genes. A Lac$^+$ recombinant plasmid, pMI400, was introduced into MC1000 with pACYC-184, pMI370 or pMI380. By monitoring the activity of β-galactosidase with different plasmids in *trans*, an additional effect of the wild-type or mutated π protein on the *pir-lacZ* expression could be analyzed. As presented in Table II, pACYC184 vector plasmids do not have any effect on this expression. However, when pMI370 which carries the wild-type *pir* gene was present in the same cells, the activity of β-galactosidase was repressed

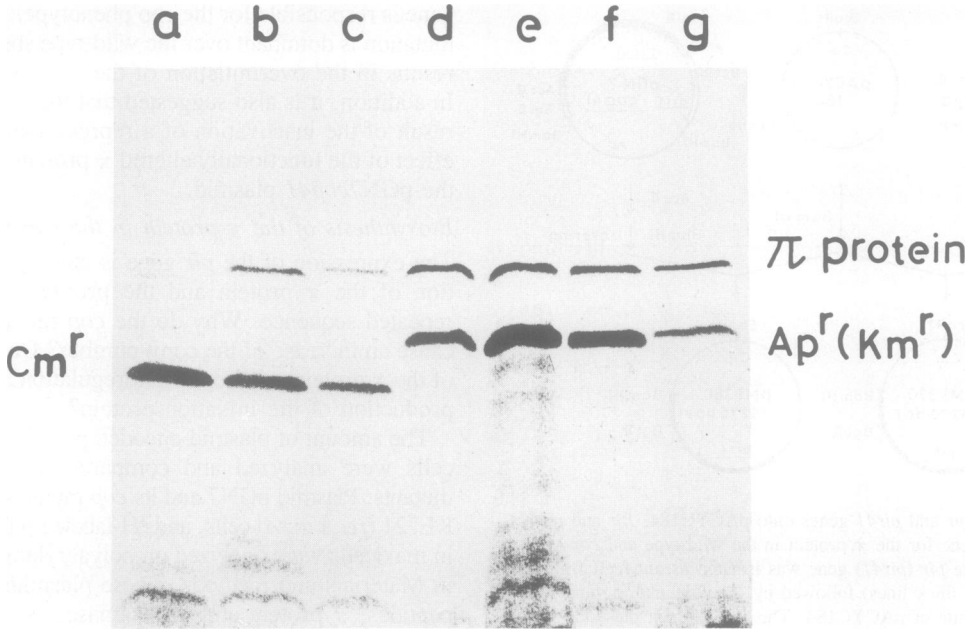


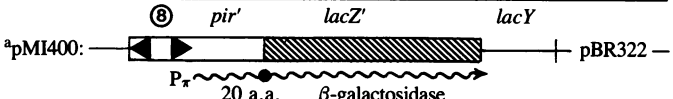
Fig. 5. Quantitative comparison of the π protein synthesized in maxicells containing the wild-type or mutant plasmids. Fluorography of ^{3}H-labeled proteins for maxicells carrying plasmids: **lanes a**, pACYC184; **b**, pMI370; **c**, pMI380; **d**, pGN7; **e**, pGN7*cop41*; **f**, pGN7*cop50*; and **g**, pRK419. The mol. wt. of the π protein was estimated at 35 000 daltons using standard mol. wt. protein markers. Cm^r, Ap^r and Km^r refer to the polypeptides encoded by chloramphenicol-, ampicillin- and kanamycin-resistant genes, respectively.

to 81%. Of most interest is the fact that when the *pir41* gene was supplied in *trans*, the inhibitory effect of the π protein strikingly increased, and only 28% of the activity of β-galactosidase remained related to pMI400. Another independent experiment gave the same result: the reduction of the activity to 73% and 24% in the presence of pMI370 and pMI380, respectively. In all cases, the copy-number of pMI400 was identical, and those of pACYC184, pMI370 and pMI380 were the same.

On the other hand, no repression effect was observed when β-galactosidase was produced by the induction with isopropyl-β-D-thiogalactoside (IPTG) of the intact *lac* operon. These results show that the inhibitory action by the π protein on gene expression is specifically related to the *pir* promoter. Considering that the π protein binds to the 8th repeated sequence in the *pir* promoter and represses *pir* expression, we suggest that the *cop*-π protein has a higher affinity for the repeated sequence than does the wild-type protein.

Table II. Effect of π protein on the expression of *lacZ* gene in cells harboring an operon fusion

Host	*pir-lac* fusion plasmid	*pir* plasmid[b]	β-galactosidase (units)	%
MC1000	pMC1403	None	3	0.2
MC1000	pMI400[a]	None	1323	100
	pMI400	pACYC184	1331	100
	pMI400	pMI370	1067	81
	pMI400	pMI380	367	28
W2252	−IPTG	None	17	1
	+IPTG[c]	None	1370	100
		pACYC184	1270	93
		pMI370	1450	106
		pMI380	1390	101



[b]pACYC184, *pir*$^-$; pMI370, *pir*$^+$; pMI380, *pir41*$^+$.
[c]Induced with IPTG.

Discussion

We have described here the isolation and characterization of the mutants with increased copy-numbers from a mini-R6K plasmid designated pGN7. These mutants, pGN7*cop41* and *cop50*, are maintained at a copy-number of ~100 per chromosome, whereas pGN7 has ~20 copies. The *cop* mutations were mapped, by recombination experiments, within the *pir* gene which encodes an initiation protein, π. The cloning of the *pir* gene of the *cop* mutant allowed us to show directly that the alteration in the *pir* gene leads to a Cop phenotype. In addition, nucleotide sequence analysis of the mutated π coding region identified a single base change corresponding to a single amino acid substitution in the π protein (Figure 3). From these results, we conclude that the structural alteration in the π protein results in the mutations which increase the copy-number in these *cop* mutants.

Recently, Stalker *et al.* (1983) isolated a cold-sensitive copy-number mutant (*cos405* mutation) which was recessive to the wild-type of R6K. We have also isolated high copy-number mutants (*cop3* and *cop21*) from the revertants derived from a temperature-sensitive replication mutant, pMI22 (*ts22*), which has the reduced copy-number of a mini-R6K (Inuzuka, in preparation). These mutations, including those in the present study, are particularly interesting since all of them are located within the *pir* gene. Additionally, the mutation sites on the π protein were found around a consensus sequence, a DBP sequence, for proteins which bind to DNA, as proposed by Pabo and Sauer (1984) (Figure 6). These results suggest that this domain of the π protein may participate in the specific interaction with the *oriγ* region DNA. Furthermore, it should be noted that the *cop* and *ts* mutations affecting the copy-number of the plasmid were located at random coiled regions of the π protein (Figure 6). A drastic conformational change of the protein appears to cause

the loss of its activity as the initiation protein.

Cop mutants, in addition to those of R6K presented here, have been isolated in plasmid P1 (Baumstark *et al.*, 1984), pSC101 (Armstrong *et al.*, 1984), RtsI (Kamio *et al.*,1984) and mini-F (Seelke *et al.*, 1982). These mutations were all mapped within the genes which encode plasmid-specific replication proteins. Some interesting common features of this group of plasmids are that there are direct repeated sequences consisting of 18 – 24 bp in their origin regions and they exhibit the incompatibility function. Also the expression of the genes for the replication proteins is autogenously regulated in most of the plasmids. Therefore, the *cop* mutations in the *rep* genes seem to be a specific characteristic among these replicons.

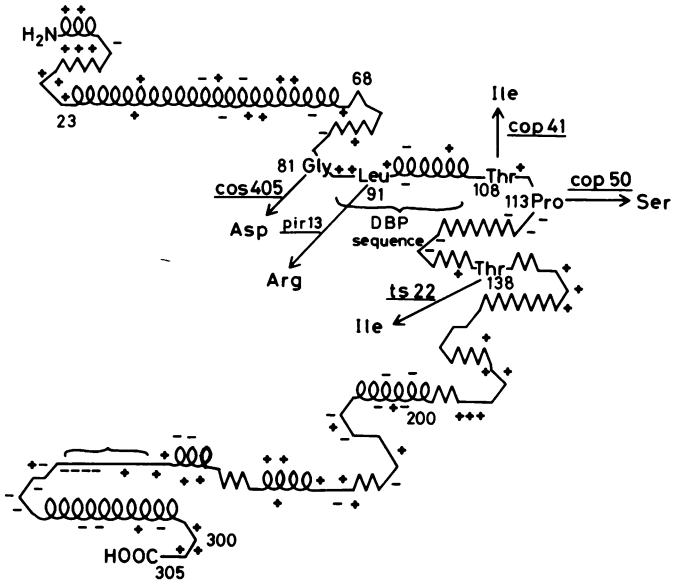


Fig. 6. Substitutions of the amino acids in the π protein by the *cop* and *ts* mutations. The predicted secondary structure of the π protein was a modification of that presented by Germino and Bastia (1982). ⌀, α-helix; ⩘, β-sheet; –, random coil; >, turn. Only the altered amino acids are shown with those in the wild-type π protein. The *cop41* and *cop50* mutations are described here; *ts22* (a temperature-sensitive) mutation is from Inuzuka (in preparation); the *cos405* mutant was isolated by Stalker *et al.* (1983); The *pir13* mutation was determined by McEachern (unpublished). The DBP sequence refers to the consensus sequence of the amino acids found in the proteins which bind to DNA (Pabo and Sauer, 1984).

To date, a *trans*-dominant copy-number mutant in the *rep* gene has not been isolated. Therefore, it should be emphasized that the *cop* mutant reported here is dominant over the wild-type π protein regarding copy control. This implies that the *cop* mutation is not due to the inactivation of a factor negatively controlling initiation. Analysis of the π protein using a maxicell system revealed that the *cop41* and *cop50* mutants never overproduced the π protein and were likely to express stronger autoregulation of the π protein synthesis (Figure 5). These results are consistent with the stronger repression of β-galactosidase synthesis in a *pir'-lacZ'* fusion operon by the *cop* mutated π protein (Table II). This means that the altered π protein has a high affinity for the repeated sequence in the *pir* promoter region. Shafferman *et al.* (1982) and Filutowicz *et al.* (1985) had observed that the expression of the π protein gene was more strongly autoregulated by the wild-type π protein than that presented here, using different *pir'-lacZ'* fusions. This effect may result from the use of the fused operons consisting of a long fragment (941 bp) on pMC81 or a 526-bp fragment of the *pir* gene on a low copy-number vector. A partially purified mutant π protein had a specific activity for R6K DNA synthesis *in vitro* which was about three times higher than that of the wild-type (unpublished). From these observations it can be readily imagined that the mutant *cop*-π protein binds more efficiently to the direct repeats, in the *ori*γ region, which are similar to the above sequence, and consequently leads to an increase in the frequency of the initiation event for DNA replication per cell division. Mutations which confer on proteins an increased affinity for specific DNA sequences have been characterized in CI repressor of phage λ (Nag *et al.*, 1984) and ρ factor of *Escherichia coli* (Tsurushita *et al.*, 1984); these are synthesized under autogenous regulation.

Thus, we suggest that a stringent conformation of the π protein is required for its normal activity as the initiation protein, and the cop phenotype is due to qualitative alterations, rather than a quantitative increase of the π protein. To determine the mechanism of action of the structurally altered π protein which is encoded by the *cop* mutants, purification of the π protein is currently under way. The relationship between the structure and function of the π initiation protein may be clarified through this approach.

Table III. Plasmids used

Plasmid	Parent replicon	Antibiotic resistance[a]	Relevant markers	Reference/source
R6K	R6K	Ap, Sm	*ori*α, β, γ, *pir*	Kontomichalou *et al.* (1970)
pRK419	R6K	Km	*ori*β, γ, *pir*	Kolter and Helinski (1978)
pMI272	pRK419, pBR322	Ap	*ori*γ, *pir*	This study
pGN7	pMI272	Ap	*ori*γ, *pir*	This study
pGN7*cop41*	pGN7	Ap	*ori*γ, *pir41*	This study
pGN7*cop50*	pGN7	Ap	*ori*γ, *pir50*	This study
pRK526	pRK419	Km	*ori*γ plasmid	Kolter *et al.* (1978)
pMI7	pGN7*cop41*	Ap	*ori*γ plasmid	This study
pBR322	pMB1	Ap, Tc		Bolivar *et al.* (1977)
pACYC184	P15A	Cm, Tc		Chang and Cohen (1978)
pMI370	pACYC184	Cm	*pir*	This study
pMI380	pACYC184	Cm	*pir41*	This study
pDS1	pACYC184	Cm		Stalker (unpublished)
pMC1403	pBR322	Ap	Lac$^-$	Casadaban *et al.* (1980)
pMI400	pMC1403	Ap	Lac$^+$	This study

[a]Abbreviations used to indicate to which antibiotics the plasmids specify resistance: Ap: ampicillin, Sm: streptomycin, Km: kanamycin, Cm: chloramphenicol, Tc: tetracycline.

Materials and methods

Bacterial strains, plasmids and media

E. coli K12 YS1, MV12(*recA trpE5*) and φ1(λ-*pir*$^+$), and *E. coli* C C2110 (*polA his rha*) were used (Kolter *et al.*, 1978; Inuzuka and Helinski, 1978a, 1978b). Strain KE721 (*recA99 uvrA trp str*) was used for maxicell experiments and kindly supplied by Dr T.Ogawa. W2252 and MC1000 (Δ(*lac*IPZOY)X74) (Casadaban *et al.*, 1980) were used for the assay of β-galactosidase. The cells were grown in L broth (10 g polypeptone, 5 g yeast extract, 5 g NaCl, 1 g glucose and 1 ml of 5 M NaOH per liter). MacConkey lactose plates were prepared as described by the supplier (Daigo Eiyo, Osaka). Antibiotics to select drug-resistant cells were added into media at the following concentrations, unless otherwise specified: penicillin G, 500 μg/ml; kanamycin sulfate, 25 μg/ml; and chloramphenicol, 25 μg/ml.

Plasmids used in this work are listed in Table III.

Enzymes and chemicals

Restriction endonucleases, T4 DNA ligase and T4 polynucleotide kinase were obtained from Takara Shuzo (Kyoto, Japan) and New England Biolabs (Beverly, Mass.). Bacterial alkaline phosphatase was from Worthington Biochemicals. Reaction conditions for these enzymes were those recommended by the suppliers.

IPTG was purchased from Sigma; *o*-nitrophenyl-β-D-galactoside was from Nakarai Kagaku (Kyoto). Synthetic *Bam*HI oligonucleotide linker (decamer) was from Takara Shuzo. Sodium ampicillin and methicillin were from Sigma.

Plasmid DNA manipulation

Plasmid DNA was prepared essentially by the cleared lysate method (Kahn *et al.*, 1979). Plasmid DNA fragments were isolated by electrophoresing on 5% polyacrylamide gels or low melting point agarose gels and eluting as described (Maxam and Gilbert, 1980; Wieslander, 1979). The sizes of DNA fragments were calibrated using pBR322 DNA digested with *Hae*II or *Hae*III and λ DNA digested with *Hind*III. DNA ligation with T4 DNA ligase was performed at 15°C in 50 mM Tris (pH 7.8); 5 mM $MgCl_2$, 0.5 mM ATP, 5 mM dithiothreitol in the presence of 7% (w/v) polyethylene glycol 6000 (Pheffer and Zimmerman, 1983).

Transformation with plasmid DNA was according to Cohen *et al.* (1972).

Construction of pGN7

Firstly to construct a mini-R6K carrying the β-lactamase gene, the *Hae*III fragment carrying the *ori*γ and *pir* genes was isolated from pRK419. After the ligation with *Bam*HI-linkers, the resultant product of 2095 bp was inserted into the *Bam*HI site of pBR322 to yield plasmid pMI272. Meanwhile, the *Bam*HI-Ap^r fragment was prepared by treatment with S1 nuclease followed by *Bam*HI-linkering of the *Hae*II-Ap^r fragment (1868 bp) from pBR322. The *Bam*HI-*ori*γ-*pir* fragment from pMI272 was ligated with the *Bam*HI-Ap^r fragment and then transformed into C2110 cells. A recombinant plasmid, pGN7, is 3963 bp (Figure 1) and its complete nucleotide sequence is known (Stalker *et al.*, 1982; Sutcliffe, 1979).

Hydroxylamine mutagenesis in vitro

The mutagenesis procedure was a modification of that previously described (Hashimoto and Sekiguchi, 1976). pGN7 DNA (5 μg) was incubated at 37°C for 21−70 h in 0.05 M sodium phosphate buffer (pH 6.0) and 1 mM EDTA with 0.4 M hydroxylamine. After a suitable interval, the mixture was withdrawn and dialyzed against 20 mM Tris (pH 7.5), 1 mM EDTA and 20 mM NaCl to stop the reaction. DNA treated with hydroxylamine for 41 h yielded Ap^r transformants which were reduced to ~20% compared with those by untreated DNA.

Estimation of the plasmid copy-number

Plasmid DNA prepared by the cleared lysate method was linearized with restriction endonucleases, electrophoresed on 1.2% agarose gels, and stained with ethidium bromide. The plasmid copy-numbers of the mutants were then estimated by densitometer tracings of film negatives taken of the agarose gels. Plasmid pDS1 was used as an internal standard.

DNA sequencing

Nucleotide sequence was determined according to the method of Maxam and Gilbert (1980). The 5′ ends of the DNA fragments were dephosphorylated with bacterial alkaline phosphatase and labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. The labeled fragments were cleaved with suitable restriction enzymes, separated on 6% polyacrylamide gels and eluted. Chemical degradation reactions specific for G, A, T + C and C were performed. The samples were analyzed on 8% and 20% sequencing gels.

Protein analysis in maxicells

Maxicell experiments were essentially performed according to the method of Sancer *et al.* (1981). Cultures of KE721 harboring the various plasmids were grown in M9-casamino acid-glucose medium containing 40 μg/ml of tryptophan and the appropriate antibiotics at 37°C until the optical density at 660 nm reached ~0.6. 5 ml of the culture was transferred into a sterile Petri dish and irradiated with u.v. light for 20−30 s at 550 μW/cm^2. After addition of 1 ml of the fresh medium, the irradiated culture was incubated at 37°C for 1 h followed by the addition of D-cycloserine at a concentration of 250 μg/ml and incubation was continued for an additional 20 h. Cells were then collected by centrifugation, washed twice with minimal M9 medium and resuspended in 2 ml of the minimal M9-salt-glucose containing tryptophan. After the cells were pre-incubated at 37°C for 60 min, the cell suspension was incubated for 60 min together with 100 μCi of L-[4,5,^{3}H]leucine (Amersham). The labeled cells were pelleted, washed three times with the minimal medium and resuspended in 50 μl of 5 mM Tris (pH 8.0)-10 mM EDTA. 5 μl of 10 mg/ml of lysozyme was added and the suspension was kept at 0°C for 10 min. After freezing of the cells in a dry ice-ethanol bath and thawing was repeated twice, an equal volume of 2 X lysing buffer (Laemmli, 1970) was added. Labeled cells were lysed by heating at 100°C for 3 min and loaded on 12.5% SDS-polyacrylamide gels according to the published procedure (Laemmli, 1970). After separation of proteins, the slab gels were treated with EN^{3}HANCE (New England Nuclear) and dried for fluorography.

β-Galactosidase assay

Strains carrying the fusion plasmids were grown in minimal casamino acid medium and assayed for β-galactosidase (Miller, 1972).

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