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An initiator protein for plasmid R6K DNA replication

Mutations affecting the copy-number control

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Two kinds of mutations affecting the copy-number control of plasmid R6K were isolated and identified in an initiator π protein by DNA sequencing. Firstly, a temperature-sensitive replication mutation, *ts22*, with decreased copy number results in a substitution of threonine to isoleucine at position 138 of the 305-amino-acid π protein. Secondly, a high-copy-number *(cop21)* mutant was isolated from this *ts* mutant and was identified by an alteration of alanine to serine at position 162. This *cop21* mutation suppressed the Ts character and was recessive to the wild-type allele in the copy control.

Plasmid R6K; DNA replication; Initiator protein; Copy mutant; Temperature-sensitive mutation; Nucleotide sequence

1. INTRODUCTION

Plasmids are maintained at a characteristic copy number in growing populations of bacteria. This copy-number control is determined mainly by a plasmid-specific gene(s) that regulates the frequency of initiation of replication per cell division. A replicon-specific initiation protein plays an especially critical role in the reaction [1].

We have studied the mechanism of control of initiation of replication by using an antibiotic-resistant plasmid, R6K. Plasmid R6K is 38 kilobase pairs in size and is maintained at a copy number of 15–20 per chromosome [2]. Three origins (*ori*α, *ori*β, *ori*γ) of replication are active in vivo [3,4] and in vitro [5,6]. We have found that an R6K-encoded protein, π protein, is directly required for the initiation of replication in vitro [7,8] and in vivo [9]. A structural gene, *pir*, for the π protein is located between *ori*β and -γ, and its nucleotide sequence has been determined [10,11]. It has also been observed that expression of the *pir* gene is autoregulated by the protein [12,13]. This initiation protein was shown to bind to direct repeated sequences within the *ori*γ region for replication [14].

To gain a better understanding of the role of π protein in replication and its regulation in R6K, we have isolated replication mutants with different copy numbers. One is a temperature-sensitive (*ts*) replication mutant [8]. Mutants belonging to the second class are high-copy-number (*cop*) mutations that are *trans*-dominant over the wild type in copy-number control. These mutations are mapped in the *pir* gene [15]. Here, we describe the characterization of two kinds of mutations in copy-number control in R6K: a *ts* mutant with decreased copy number and *cop* mutants isolated from the *ts* mutant. These mutations result in a single amino acid substitution in π protein, the *cop21* mutation being *trans*-recessive to the wild type.

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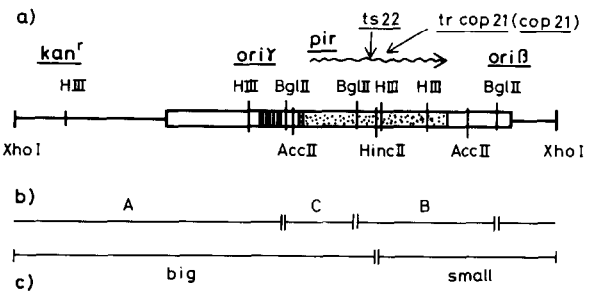

Fig.1. Physical and genetic maps of plasmid pRK419, pMI22 or pMI22*trcop21*. (a) The 2.85 kilobase pair fragment derived from R6K is shown by a double line. *oriβ* and *oriγ* refer to the initiation sites of DNA replication. Arrows indicate the sites of a *ts22* in pMI22 and *trcop21* mutations within the *pir* gene. HIII, *Hin*dIII restriction sites. (b) *Bgl*II fragments A, B, and C refer to the 2400, 1069 and 527 bp fragments, respectively, produced by digestion with *Bgl*II. (c) Large and small fragments, produced by digestion with *Xho*I and *Hin*cII, were used in fragment-mixing experiments.

2. MATERIALS AND METHODS

2.1. *Bacterial strains and plasmids*

Escherichia coli K12 strain MV12 (*recA trpE5*) was used [9]. Plasmid pMI22 is a *ts* replication mutant [8] isolated from pRK419 [9] (fig.1). pMI467 was constructed by ligation of the *Acc*II-*pir* fragment prepared from pMI22*trcop21* and pACYC184 DNA digested with *Hin*cII. The properties of pMI7, pGN7, pMI370 and pMI380 plasmids have been detailed in the preceding paper [15] and in table 2. pDS1 is a mini-pACYC184 derivative [15].

2.2. *Isolation of copy-number mutants and estimation of the plasmid copy number*

High-copy-number mutants were isolated by plating cultures of MV12(pMI22) grown at 30°C onto L agar plates containing 25 μg/ml of kanamycin sulfate and by incubation at 42°C. Cleared lysates [16] prepared from the cultures of temperature-resistant (*tr*) colonies were assayed for increased copy number by agarose gel electrophoresis. Plasmid copy numbers of the mutants were estimated as in [15]. Copy numbers of plasmids harboring the *amp*^{r} gene were also estimated by using the fact that levels of resistance to ampicillin are dependent on the *amp*^{r} gene dosage, i.e. proportional to plasmid copy number [15,17].

2.3. *DNA sequencing, media, enzymes and DNA manipulation*

Nucleotide sequences were determined according to Maxam and Gilbert [15,18]. The other procedures have been described [15].

3. RESULTS

3.1. *Identification of the site of* ts *mutation in pMI22*

The mutant plasmid, pMI22, temperature-sensitive for replication, that can be maintained at 30 but not 42°C, was isolated from pRK419 by hydroxylamine mutagenesis in vitro. The copy number of this plasmid was about half of that of pRK419 even at a permissive temperature, as shown in fig.2.

We first performed fragment-mixing experiments in which three *Bgl*II fragments of pMI22 were tested for their ability to express the Ts phenotype, by replacing the corresponding pRK419 fragments. As shown in table 1, the *ts* mutation was mapped on the medium-sized *Bgl*II fragment (B) of 1069 base pairs in which the coding region for position 117 to the COOH-terminal amino acid of the protein is included. This fragment was then sequenced and the result compared to the sequence of pRK419. A single base exchange from C to T was observed at position 868 of the wild-type *pir* sequence [11,12] (fig.3). This change occurred in the 2nd position within the codon (ACC to ATC) and resulted in the change from threonine to isoleucine at position 138 of the π protein.

3.2. *Isolation of high-copy-number mutants from pMI22*

cop mutants from pMI22 were isolated by selecting spontaneous revertants that could grow at 42°C, as described in section 2. Colonies carrying temperature-resistant (Tr) revertants with increased copy numbers were found at a frequency of approx. 3×10^{-9} per parent cell. Independent *cop* mutants were designated pMI22*trcop1*, *cop3* and *cop21*. The copy numbers of these mutants increased at least 4-, 8- and 7-fold for *cop1*, *cop3* and *cop21* mutants, respectively, in comparison with 20 copies of pRK419 (fig.2). The genetic and biochemical properties of the *cop21* mutant were further studied.

3.3. *Localization of the* cop21 *mutation in the* pir *gene*

The site of *cop21* mutation was located on the same *Bgl*II fragment (B) in which the site of *ts* mutation was determined through fragment mixing (table 1). By DNA sequencing of this fragment, a single base substitution from G to T was found at base pair position 938 within the *pir* gene. This change was located in the first position of the 162nd codon of the *pir* gene and resulted in a single amino acid alteration from alanine (GCA) to

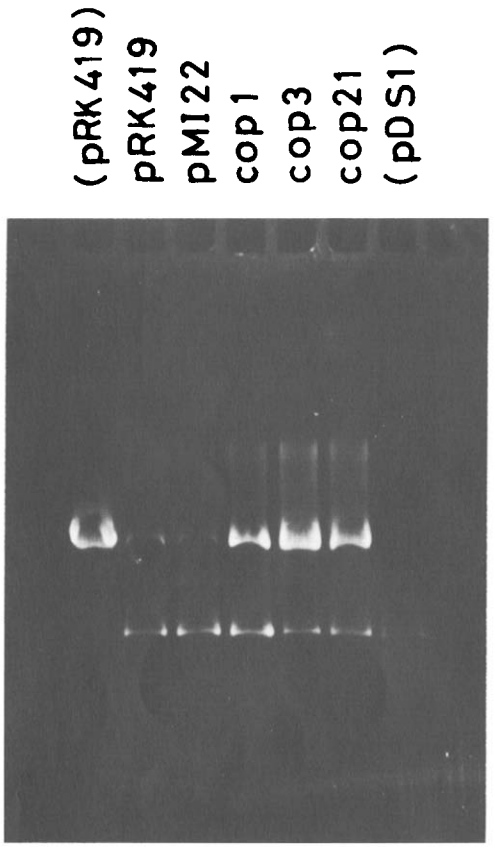

Fig.2. Copy number of pRK419, pMI22 and its *cop* mutants in MV12 (pDS1). DNAs were prepared by the cleared lysate method and digested with *Xho*I and *Eco*RI to linearize pRK419 derivatives and pDS1 DNA, respectively. (pRK419) and (pDS1) on either side of the samples refer to linear molecules of each plasmid. pDS1 was used as an internal standard to test the efficiency of DNA extraction.

serine (TCA) (fig.3). Compared to the sequence of pRK419, in the pMI22*trcop21* mutant the *ts22* mutation was still conserved at nucleotide 868. No base change was found in the remainder of the *pir* gene including the operator region. Therefore, we conclude that the single amino acid alteration in the π protein results in both the reversion of the Ts character and the mutation which increases the copy number. It was also shown from the fragment-mixing experiments that the Cop phenotype is the result of only one *cop21* mutation and is not necessary for the *ts22* mutation: the recombinant plasmid of a small *Hin*cII-*Xho*I fragment of pMI22*trcop21* and a large one from pRK419 (figs 1,3) expressed both Tr replication

Table 1

Analysis of the *ts22* and *trcop21* mutations by fragment-mixing experiments

(1) Recombination with *Bgl*II fragments[a]			Replication of recombinants	Relative copy number[b]
A	B	C		
ts	ts	ts	Ts	low
Wild	wild	wild	Tr	ordinary
trcop21	trcop21	trcop21	Tr	high
Wild	wild	ts	Tr	ordinary
Wild	ts	wild	Ts	low
ts	wild	wild	Tr	ordinary
Wild	wild	trcop21	Tr	ordinary
Wild	trcop21	wild	Tr	high
trcop21	wild	wild	Tr	ordinary
(2) *Xho*I-*Hin*cII fragments				
Large		Small		
Wild		wild	Tr	ordinary
trcop21		trcop21	Tr	high
Wild		trcop21	Tr	high
trcop21		wild	Ts	low

[a] Wild, ts and trcop21 DNA fragments were prepared from pRK419, pMI22 and pMI22*trcop21*, respectively, as shown in fig.1

[b] Estimated by densitometer tracings of film negatives taken of agarose gels of the cleared lysates

and Cop phenotype, while a plasmid constructed in the reverse combination was Ts and at a low copy number (table 1).

3.4. *The* cop21 *mutation is recessive to the wild type*

To clarify whether the *cop* mutation is dominant or recessive to the wild-type allele in the copy control, the *cop21* mutated *pir* gene (*pir21*) was cloned onto a vector pACYC184 as described in section 2. The resultant plasmid, pMI467, was then introduced into MV12 cells carrying pMI7, pGN7 or pMI22*trcop21*, and the copy numbers of R6K replicon plasmids and resistance levels to ampicillin were analyzed (table 2). As characterized *pir* plasmids, pMI370 and pMI380 harboring the wild type *pir*$^+$ and *trans*-dominant *cop* mutated *pir41*$^+$ genes, respectively, were used [15]. When the *cop21*-mutated π protein produced by pMI467 was supplied in *trans*, the *ori*γ plasmid, pMI7, exhibited resistance to high concentrations of am-

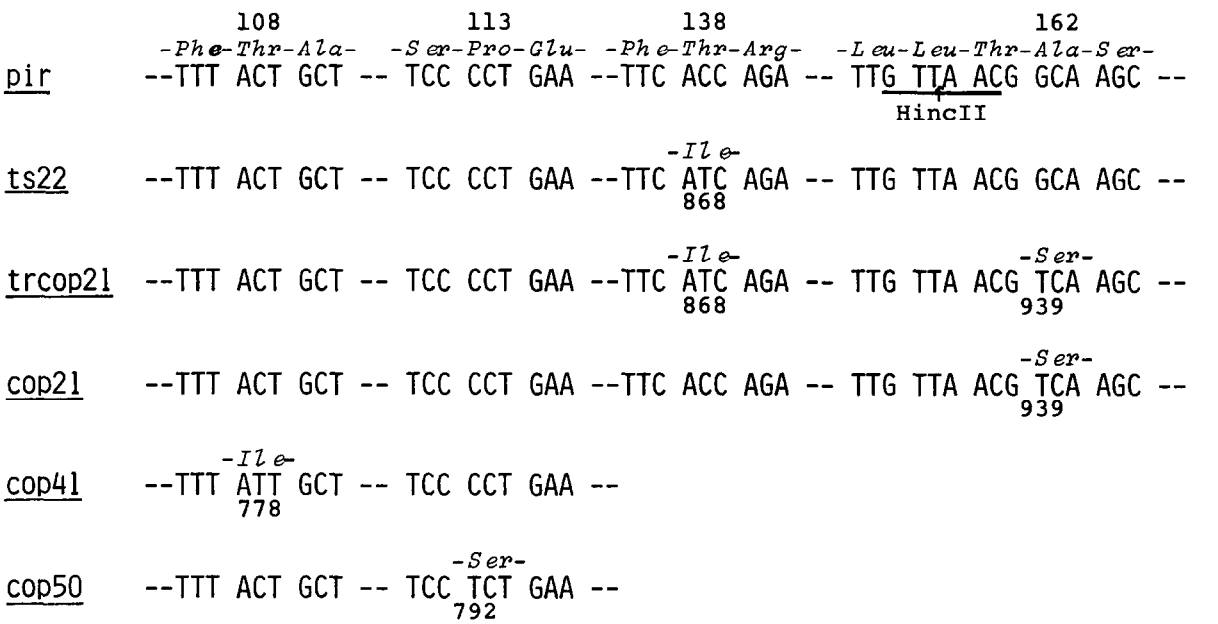

Fig.3. Nucleotide sequences of *pir* gene containing *ts* and *trcop* mutations. Only relevant regions close to mutation sites are shown. Numbers above amino acids refer to altered amino acids in the 305-amino-acid π protein. Numbers below bases indicate mutated bases. Nucleotide 1 corresponds to the *Hin*dIII restriction site in the *ori*γ region. The *cop41* and *cop50* mutations are described in [15].

picillin and methicillin and the same value for increased copy number as that in the presence of pMI380. On the other hand, when pMI467 coexisted with pGN7 which is a mini-R6K carrying the wild-type *pir*$^+$, *ori*γ and *amp*^{r} genes, pGN7 maintained its own characteristics in the copy control. pMI380 harboring the *trans*-dominant *pir41* gene increased the copy number of pGN7. Moreover, the copy number of pMI22*trcop21* decreased to the ordinary value with coexisting pMI370. These results indicate that the *cop21* mutation is recessive to the wild type regarding the copy control.

Table 2

Effect of co-existing *pir* plasmids on copy number of R6K plasmids

R6K plasmid[a]	*pir* plasmid	Sensitivity[c] to A_6M_4	Copy number[d]
pMI7	pMI467	R	high
pMI7	pMI370	S	ordinary
pMI7	pMI380	R	high
pGN7	pACYC184	S	ordinary
pGN7	pMI467	S	ordinary
pGN7	pMI370	S	ordinary
pGN7	pMI380	R	high
pMI22*trcop21*	pACYC184		high
pMI22*trcop21*	pMI370		ordinary

[a] pMI7, *ori*γ-*amp*^{r}; pGN7, *ori*γ-*pir*$^+$-*amp*^{r}; pMI22*trcop21*, *ori*γ-*pirtrcop21*$^+$-*kan*^{r}

[b] pMI467, pACYC184-*pir21*$^+$; pMI370, pACYC184-*pir*$^+$; pMI380, pACYC184-*pir41*$^+$

[c] A_6M_4, ampicillin (6 mg/ml) and methicillin (4 mg/ml) in L-agar plates

[d] Estimated as described in table 1

4. DISCUSSION

We have identified two kinds of mutations which alter the function of the protein for the regulation of R6K DNA replication. One exhibits a Ts replication phenotype by substituting a single amino acid in the π protein and results in a reduction of copy number of the plasmid even at a permissive temperature. These results are consistent with the instability of the mutated π protein in vitro [8]. The second type is that of mutants with increased copy numbers which are isolated from the *ts* mutant. These also suppress the Ts character of the π protein. This result suggests that structural alteration of the protein, at or near the amino acid substituted with the *cop21* mutation, may affect

the folding of the Ts π protein, 23 amino acids away, at the site of the *ts22* mutation. Analogous mutants were isolated in pSC101 [19]. In addition to those reported here, four *cop* mutations in the *pir* gene have been identified by means of the nucleotide sequences, namely *cos405, pir13, cop41* and *cop50* alter the 81st, 91st, 108th and 113rd amino acid of the 305-amino-acid π protein, respectively [15,20]. Mutations affecting the copy control are all within a region 82 amino acids in length that contains consensus sequences [21] for DNA-binding protein. Therefore, one can imagine that a copy-control domain of the π protein is located in its central region. The fact that the *cop21* mutant is recessive to the wild type as shown in table 2 can be explained by postulating that the active π protein is a dimer and that a molecule composed of a mutant subunit and a wild-type subunit has wild-type activity. On the other hand, subunits of the *trans*-dominant *cop41*-mutated π protein may have a greater affinity for forming the active dimer than in the case of combination with the wild type subunit. It is most interesting that *cop* mutants of the initiation protein have been isolated in other plasmids, viz. pSC101, miniF, P1 and Rts1 which have direct repeated sequences in their origin regions as has R6K [19,22–24].

Using these *cop* mutants and considering that the π protein binds to the direct repeats in the *ori*γ region for initiation [14,25], further experiments are currently underway in order to reveal how the π protein regulates the frequency of initiation of R6K DNA replication.

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