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A New Gene of Bacteriophage P4 That Controls DNA Replication

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Bacteriophage P4 replication may result in either a lytic cycle or plasmid maintenance, depending on the presence or absence, respectively, of helper phage P2 genome. Bacteriophage P4 DNA replication depends on the product of gene α, which has origin recognition, primase, and helicase activities. An open reading frame with the coding capacity for a protein of 106 amino acids (*orf106*) is located upstream of the α gene. Genes *orf106* and α are transcriptionally coregulated. Three amber mutations and an internal deletion (*del51*) were introduced into *orf106*. All of the amber mutations exhibited a polar effect on transcription of the downstream α gene. The P4 *del51* mutant was slightly defective in lytic growth and could not be propagated in the plasmid state. In this latter condition, P4 DNA overreplication was observed. Overexpression of Orf106 severely inhibited P4 DNA replication, preventing P4 lytic growth and plasmid maintenance. The inhibitory effect of Orf106 on P4 replication was not observed when both *orf106* and α were overexpressed. We suggest that *orf106* is involved in P4 replication and that a balanced expression of *orf106* relative to α may be necessary for proper P4 DNA replication. In particular, *orf106* appears to be essential for the control of P4 genome replication in the plasmid state. We propose that *orf106* be named *cnr*, for copy number regulation.

P4 is a temperate bacteriophage that lacks morphogenetic and lysis functions and depends on a helper phage, such as P2, for the lytic cycle. In the absence of P2, P4 can propagate in the plasmid state. Both in the presence and in the absence of the helper, P4 can lysogenize the bacterial host *Escherichia coli* (for a review, see reference 18).

P4 DNA replication is independent of the helper bacteriophage. Three elements of P4 are known to be essential for its DNA replication: two *cis*-acting regions, the replication origin (*ori*) and the *cis* replication region (*crr*), and the *trans*-acting gene α, whose product has origin recognition, primase, and helicase activities (9, 15, 29). Protein α binds in vitro both to *ori* and *crr*; however, replication starts exclusively at the *ori* site (15, 29).

α is the last gene of an early transcription unit (α operon; Fig. 1). Immediately upstream of the α gene is an open reading frame called *orf106*, which has the capacity to encode a protein of 106 amino acids (9). A protein of this approximate size has been observed after infection of maxicells (1) and minicells (12). Furthermore, by cloning the P4 4260-7652 DNA fragment into an expression vector, an overproduced protein that corresponds well to the predicted size for the *orf106* protein could be detected after induction (6).

The α operon is transcribed from two promoters: the constitutive promoter P_{LE}, from which α is expressed immediately after infection, and P_{LL}, located 400 bp upstream of P_{LE}, which is activated late after infection and in the plasmid state (7, 8). Transcription from these promoters generates transcripts covering the whole operon (4.1- and 4.5-kb transcripts, starting from P_{LE} and P_{LL}, respectively) ending at the tα termination site, located immediately downstream of α, and transcripts ending at a termination site, t151, located immediately upstream of *orf106* (1.3- and 1.7-kb transcripts [7, 8, 26]). Thus, genes α and *orf106* appear to be coregulated.

To study the function of *orf106*, we created suppressor-sensitive and deletion mutations in this gene; in addition, we tested the effect on P4 DNA replication of the overexpression of *orf106*. Our results suggest that *orf106* regulates P4 replication and that an imbalance of *orf106* gene expression relative to α alters P4 copy number control.

MATERIALS AND METHODS

Bacterial and phage strains. All strains used are derivatives of *E. coli* C. C-1a is prototrophic (22), and C-5538 is its P2 *lg* lysogenic derivative; C-5204 is a P2 *lg* lysogenic polyauxotrophic strain (11). Strain C-2421 is a derivative of C-1a in which Δ(*argF-lac*)*U169* of strain SH210 (23) had been transduced by P1, and F-*proAB lacI*^{q} *ZΔM15* Tn*10* from strain XL1-Blue (5) was transferred by conjugation (14).

Previously described bacteriophages used are P2 *lg* (3), P4 (24), and P4 *vir1* (19). P4 *vir1 orf106am1*, *-am2*, and *-am3*, P4 *orf106del51*, and P4 *vir1 orf106del51* are described in this work.

Plasmids. The following plasmids were derived from pUC19 (28) by insertion of a P4 DNA fragment in the polylinker region. pFHE1 carries the P4 *Sma*I (position 5849)-*Sph*I (position 7631) region; pFHE2A carries the same P4 fragment with the *orf106am1* mutation; pGM276 contains the *Bam*HI (4260)-*Sph*I (7631) P4 region, and pGM290 carries the same fragment with an internal *Hin*cII (5253 to 6447) deletion; pGM283 carries the *Not*I (6726)-*Sph*I (7631) region. pFHE4 contains the *Hin*dIII (3687 to 10442) fragment of P4 ligated into pRB30, a derivative of pBR322 (4) modified by *Sph*I digestion, filling with DNA polymerase I Klenow fragment, and religation to eliminate the *Sph*I restriction site. P4-6 has the *Eco*RI fragment of P4 *vir1* (3631 to 220, containing *cos*) cloned into pBR322 cut with *Eco*RI.

Construction of the *orf106am* mutations. The *orf106am* mutations were constructed by oligonucleotide-directed site-specific mutagenesis by the method of Kunkel (16), modified as

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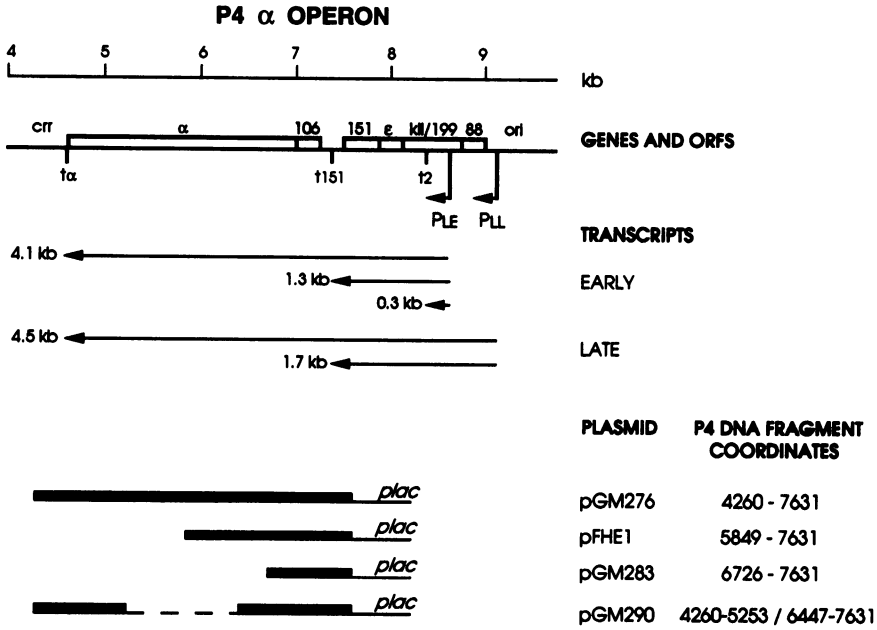

FIG. 1. Map of the α operon, illustrating the main features of the P4 α operon as identified by Halling et al. (13) and Dehò et al. (8). The P4 DNA fragments cloned in the plasmids are indicated.

described by Van Bokkelen et al. (27). A *Hin*cII (6447)-to-*Sph*I (7631) fragment of P4 was cloned into pUC118 (28) for mutagenesis. The *am1* mutation is a GC-to-CG transversion at position 7209 that changes a tyrosine codon to amber; *am2* is an AT-to-CG substitution at position 7149 that changes a tyrosine codon to amber; *am3* is a GC-to-TA transversion at position 7057 that changes a serine codon to amber (Fig. 2). Each of the three mutated fragments, following verification by DNA sequencing, was cloned into pFHE1 to replace the wild-type *Hin*cII (6447)-to-*Sph*I (7631) P4 sequence. From the resulting plasmids, the mutant P4 *Sma*I (5849)-to-*Sph*I (7631) fragments were excised and cloned into pFHE4 to replace the wild-type *Sma*I (5849)-to-*Sph*I (7631) P4 sequence. From these plasmids, the mutant *Hin*dIII (3687 to 10442) P4 fragments were excised and cloned into P4-6. The large *Eco*RI fragments containing the *orf106am* mutations were excised and ligated with the complementary fragment (220 to 3631) from a partial *Eco*RI digest of P4 *vir1*.

Construction of the *orf106del51* mutation. The *del51* deletion was constructed in two steps. First, the *Bam*HI (4260)-*Stu*I (7945) P4 DNA fragment was cloned in pUC19 cut with *Bam*HI and *Hin*cII (plasmid pGM274). Then three different fragments of pGM274 were purified and ligated: (i) *Hin*dIII-*Not*I (6726); (ii) *Not*I (6726)-*Ava*I (7041), in which the *Ava*I site had been filled with polymerase I Klenow fragment; and (iii) *Hin*dIII-*Hin*fI (7189), in which the *Hin*fI site had been filled. The resulting plasmid (pGM289) was sequenced to confirm the deletion of nucleotides 7046 to 7189 (*del51*). To transfer the mutation into both P4$^+$ and P4 *vir1* genomes, the *Sma*I (5849)-*Sph*I (7631) fragment of pGM289, containing *del51*, was ligated with the P4 *Mlu*I (8622)-*Sph*I (7631) fragment and with the *Mlu*I (8622)-*cos*-*Sma*I (5849) fragments of P4$^+$ and P4 *vir1*, respectively.

One-step growth. The bacterial strains were grown in LD broth (12) supplemented with 5 mM $CaCl_2$ and 100 μg of ampicillin per ml, when needed, to an optical density equivalent to 10^8 cells per ml. The cells were infected at a multiplicity of infection of 10 to 20 and incubated at 37°C. After 10 min, phage adsorption was measured, and P2 antiserum (K = 2.5 min^{-1}) was added to eliminate the unadsorbed phage. At 20 min after infection, the cultures were assayed to determine the titer of cells surviving the infection and of cells yielding phage.

```
                    7300
                     |
CAGGGAGAAAAGCAAATGAAAACACCCTTACCGCCCGTCTTACGCGCTGCC
    SD          M  K  T  P  L  P  P  V  L  R  A  A
                orf106 -->

CTTTACCGTCGCGCTGTCGCCTGTGCCTGGCTGACCGTGTGCGAACGTCAG
L  Y  R  R  A  V  A  C  A  W  L  T  V  C  E  R  Q

            7200
      Gam1   |                          del51
CACCGCTACCCGCATCTCACCCTTGAGTCACTGGAGCGCGGCATCGCCGCT
H  R  Y  P  H  L  T  L  E  S  L  E  A  A  I  A  A

                        Gam2
GAGCTGGAAGGCTTTTATCTGCGCCAGCACGGTGAGGAAAAAGGGCGTCAG
E  L  E  G  F  Y  L  R  Q  H  G  E  E  K  G  R  Q

            7100
             |
ATAGCCTGTGCCCTGCTGGAAGATTTAATGGAATCCGGCCCCCTGAAGGCC
I  A  C  A  L  L  E  D  L  M  E  S  G  P  L  K  A

      Aam3
GCGCCGTCGCTGTCCTTTCTCGGGCTGGTTGTGATGGATGAACTCTGTGCC
A  P  S  L  S  F  L  G  L  V  V  M  D  E  L  C  A

          7000
           |
CGTCACATAAAAGCGCCGGTACTGCACTGAAGGAGAAACCCAACATGAAA
R  H  I  K  A  P  V  L  H  Stop SD          M  K
                                            α -->
```

FIG. 2. *orf106* sequence and mutations. The sequence of the sense strand is shown from nucleotides 7322 to 6964. The coordinates are from the complete P4 sequence (13) in the revised form available from GenBank (accession number X51552). The base substitutions present in *orf106am1*, -am2, and -am3 are indicated above the sequence in boldface. The nucleotides missing in *del51* are overlined.

Phage production was assayed by using a sample which was diluted 10^4 times in LD and incubated at 37°C with aeration for 120 min.

P4 maintenance in the plasmid state. Cell growth, infection, and measurement of the survivors were carried out as in the one-step growth experiment. The colony morphology of the surviving clones was used to detect the pP4 carriers; pP4 carrying clones exhibit a rosette colony morphology, whereas P4 lysogenic cells or cells that do not carry P4 have a normal colony morphology (1). The presence of either P4 prophage or pP4 was confirmed by qualitative *cox* test analysis (1) of 24 surviving clones for each infection: pP4 carriers produce P4 following infection with P2 *cox*, whereas P4 lysogens are induced only by P2 cox^+.

Northern (RNA) blot hybridization. RNA was extracted from uninfected *E. coli* and from the P4-infected cells as described by Dehò et al. (7), fractionated by 1.5% formamide–formaldehyde agarose denaturing gel electrophoresis, transferred onto Hybond-N filter membranes (Amersham) by vacuum blotting, and hybridized to the P_{LE}-t2 riboprobe as described by Dehò et al. (8). The P_{LE}-t2 riboprobe was produced by polymerase SP6 transcription of pGM141 digested by *Rsa*I (8); it covers P4 coordinates 8418 to 8774.

Total DNA extraction and Southern blot hybridization. Total DNA was extracted from a 2-ml sample of the infected cultures at an optical density of 50 Klett units (about 10^8 cells per ml) as described by Ljungquist and Bukhari (20). The total DNA concentration determined spectrophotometrically was equalized when necessary; the DNA was digested to linearize P4 DNA and separated by 0.6% agarose gel electrophoresis. Southern blot hybridization of the DNA transferred by vacuum blotting onto Hybond-N filter membranes with the P_{LE}-t2 riboprobe was carried out at 42°C as described by Sambrook et al. (21).

RESULTS

Construction of mutations in *orf106*. To study the role of the *orf106* gene, three different amber mutations were constructed in the *orf106* sequence (see Materials and Methods). These are base substitutions at 7209, 7149, and 7057 that create amber mutations in codons 32 (*am1*), 52 (*am2*), and 83 (*am3*), respectively of *orf106* (Fig. 2). The mutations were transferred into P4 *vir1* DNA, and the phenotypes of the mutant phage were analyzed. The presence of the amber mutations reduced P4 lytic growth, inhibited the maintenance in the plasmid state, and impaired P4 DNA replication (data not shown). This phenotype might be ascribed to a polar effect of the amber mutations on the α gene, which is necessary for P4 DNA replication. In fact, the growth defect of P4 *orf106am* mutants was suppressed in a polarity suppressor *suA* (Rho^- [17]) mutant host (data not shown). Furthermore, the transcription pattern of the α operon was altered in the P4 *vir1 orf106am* mutants: the 4.1- and 4.5-kb transcripts, which cover the full operon, were produced in reduced amount, whereas the shorter transcripts from the same operon, ending upstream of *orf106*, were present in normal quantity (Fig. 3). This finding suggests that the amber mutations in *orf106* may cause premature termination of downstream transcription and/or instability of the long transcripts.

To avoid the polar effects on α gene expression of an amber mutation in *orf106*, we constructed a mutant in which 47 internal codons of the *orf106* gene were deleted without altering the reading frame (*del51* in Fig. 2; for construction, see Materials and Methods). The deletion was transferred into both $P4^+$ and P4 *vir1*. Northern analysis revealed that transcription of the α operon was not altered in the mutant phage. In particular, the 4.1- and 4.5-kb RNAs were present in normal amounts (data not shown). Therefore, the deletion does not cause polarity.

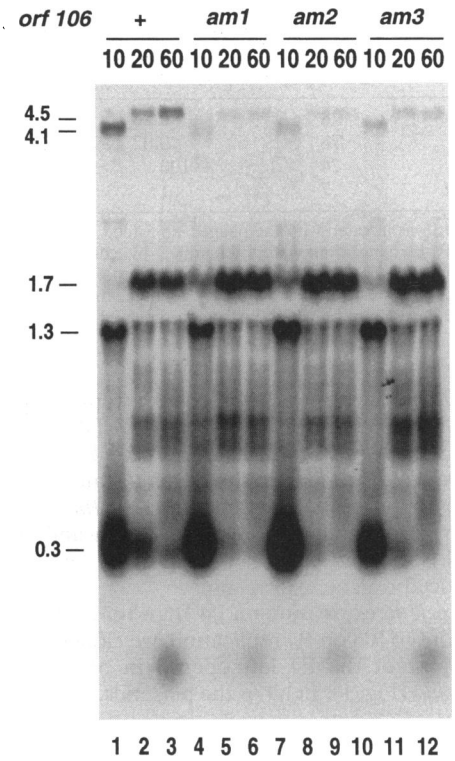

FIG. 3. Transcripts of the α operon synthesized by P4 *vir1 orf106am* mutants, identified by Northern blot analysis of RNA extracted from C-1a 10, 20, and 60 min after infection with the P4 mutants listed below. The RNAs were hybridized to the P_{LE}-t2 riboprobe (which covers P4 8418 to 8774 [8]). The sizes (in kilobases) of the main P4 transcripts are indicated at the left. Lanes: 1 to 3, P4 *vir1*; 4 to 6, P4 *vir1 orf106am1*; 7 to 9, P4 *vir1 orf106am2*; 10 to 12, P4 *vir1 orf106am3*.

The *del51* mutation caused a reduction of P4 lytic growth in P2 lysogenic cells (Table 1). In the absence of P2, the mutant could not be maintained in the plasmid state (Table 2). Following infection of C-1a with the double mutant P4 *vir1 orf106del51*, cell survival was low (7% of the value for infected cells), and the survivors carrying P4 were scarcely viable and segregated cured cells. In liquid medium, the growth rate of C-1a infected with P4 *vir1 orf106del51* started decreasing 2 h after the infection, concomitantly with an increase of the P4 DNA copy number. After 6 h, the culture stopped growing (Fig. 4). These data indicated that in P4 *orf106del51*, the lack

TABLE 1. One-step growth of P4 *del51*

Infecting phage[a]	MOI[b]	Survivors (%)	Cells yielding phage (%)	No. of phage/infected cell
$P4^+$	19	28	91	47
P4 *del51*	24	30	89	33
P4 *vir1*	11	0.05	>100	169
P4 *vir1 del51*	16	0.13	>100	38

[a] Infection of strain C-5538 was performed as described in Materials and Methods.
[b] MOI, multiplicity of infection.

TABLE 2. P4 propagation in the plasmid state

Infecting phage[a]	Surviving clones (%)	pP4 carriers/ survivors[b] (%)	Lysogens/ survivors[b] (%)
P4$^+$	78	0.26	96
P4 *del51*	78	<0.02	100
P4 *vir1*	74	100	<1
P4 *vir1 del51*	7[c]	<1	<1

[a] Strain C-1a was infected with the phages indicated.
[b] Plasmid P4-carrying clones were identified by colony morphology and qualitative *cox* test as described in Materials and Methods.
[c] The clones are mostly cured of the P4 genome; the few P4 carriers are scarcely viable and cannot be maintained upon repeated streaking.

TABLE 3. Effect of *orf106* on P4 efficiency of plating

Bacterial strain[a]	P4 gene(s) carried by the plasmid	Efficiency of plating[b] of:	
		P4$^+$	P4 *vir1*
C-5204/pGM283	*orf106*	$<10^{-3}$	$<10^{-3}$
C-5204/pFHE1	*orf106*	10^{-2}	10^{-2}
C-5204/pFHE2A	*orf106am1*	1	1
C-5204/pGM276	*orf106*, α, *crr*	1[c]	1
C-5204/pGM290	*orf106*, *crr*	10^{-2}	10^{-2}

[a] The bacterial strains were plated in the presence of IPTG (40 μg/ml).
[b] Relative to that of the control strain C-5204/pUC19, which was assigned a value of 1.
[c] Clear-plaque morphology.

of the *orf106* product caused P4 DNA overreplication, suggesting that *orf106* might negatively control P4 DNA replication.

Neither the P4 *vir1 orf106del51* nor the P4 *vir1 orf106am* mutants can be maintained in the plasmid state. However, this is caused by opposite defects: in P4 *vir1 orf106del51*, plasmid propagation is prevented by overreplication and cell killing, while in P4 *vir1 orf106am*, it is prevented by underreplication due to reduced α gene expression.

Effect of *orf106* expression on P4 DNA replication. To study the effects of Orf106 on P4 replication, we cloned fragments of the distal part of the P4 left operon in pUC19 under the control of *plac* (Fig. 1). In all of the plasmids, a fusion between the first codons of *lacZ* and the terminal 63 codons of *orf151* was created; thus, in these constructs, the transcription-translation signals regulating the expression of *orf106* and α were preserved.

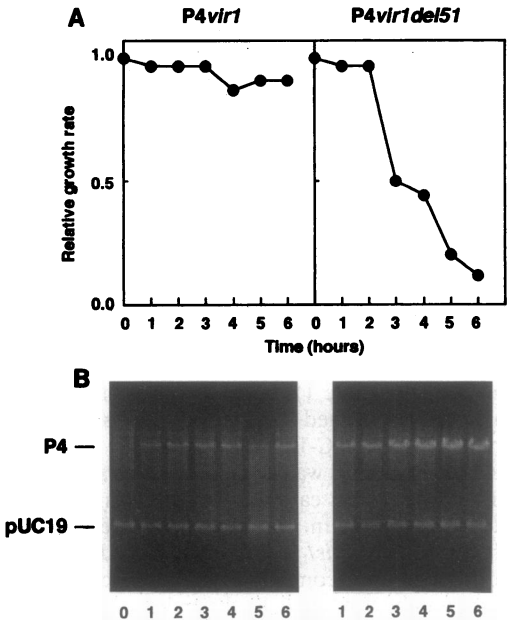

FIG. 4. P4 replication and growth rate of P4-infected cells. A culture of strain C-1a/pUC19 was infected with P4 *vir1* (left) or P4 *vir1 orf106del51* (right) at a multiplicity of infection of 10 and incubated at 37°C with aeration, and the absorbance was monitored. The cultures were kept in the exponential phase of growth by repeated dilutions whenever they reached an absorbance of 80 Klett units (about 2×10^8 cells per ml). At various time points, samples were taken and total DNA was extracted (see Materials and Methods). (A) Relative growth rates, measured as increments in absorbance of the infected cultures 1 to 6 h after infection relative to the increment measured in an uninfected culture (zero time point). (B) Agarose gel electrophoresis of DNA extracted from uninfected cells (lane 0) and from infected cells 1 to 6 h after infection. The DNA samples were digested with *Pst*I, which linearizes both P4 and pUC19. The linearized P4 and pUC19 DNAs are indicated at the left.

The efficiency of plating on P2 lysogenic strains carrying the different plasmids was measured (Table 3). Plasmids carrying the wild-type *orf106* gene without the complete α sequence (pGM283, pFHE1, and pGM290) inhibited P4 growth. This effect was not observed when the plasmid carried the *am1* mutation in *orf106* (pFHE2A). A plasmid which expresses both *orf106* and α (pGM276) did not inhibit P4 growth. In the presence of this plasmid, P4$^+$ plaques were clearer and larger than on the control strain.

In one-step growth experiments, plasmid pGM283, which expresses *orf106*, reduced the yielder frequency and the phage yield of P4$^+$ and P4 *vir1* (Table 4). This inhibitory effect was not exhibited when the α gene was coexpressed with *orf106* (pGM276). pFHE1, which carries the *orf106* gene and about half of the α gene, inhibited P4 growth less severely than pGM283 (see Discussion).

The effect of *orf106* expression on the maintenance of P4 in the plasmid state in the absence of P2 was tested. Infection of C-2421/pGM283 with P4 *vir1* in the presence of the inducer isopropylthiogalactopyranoside (IPTG) prevented the establishment of the plasmid state (Table 5). In the absence of the inducer, P4 *vir1* plasmid-carrying clones were isolated, but they were highly unstable and segregated P4 lysogenic or cured clones (data not shown), possibly because of the basal level of expression of *orf106* in the absence of the inducer. Coexpression of *orf106* and α (pGM276) did not inhibit the establishment of P4 plasmid state, although the clones obtained were scarcely viable and segregated cured or inviable subclones even if grown in the absence of the inducer IPTG (Table 5).

The interference with P4 lytic growth and with maintenance in the plasmid state observed when the cloned *orf106* was expressed from a vector might be due to inhibition of P4 DNA replication. We therefore analyzed the replication of P4 *vir1* in cells overexpressing *orf106*. A Southern blot of DNA extracted at different times following infection is shown in Fig. 5. The results obtained indicated that P4 DNA replication was inhibited almost completely by pGM283; in the presence of pGM276, not only was this inhibitory effect not observed, but also P4 replication was increased.

To test whether the inhibition of P4 DNA replication caused by *orf106* might depend on a lower expression of α, we analyzed P4 transcription in the presence of pGM283. The transcripts of the α operon synthesized by P4 *vir1* following infection of C-2421/pGM283 were analyzed by Northern blot hybridization (Fig. 6). In the first 20 min after infection, the transcription pattern of the P4 left operon was normal; thus, at

TABLE 4. Effect of *orf106* on P4 growth[a]

Bacterial strain	P4 gene(s) carried by the plasmid	Infection with P4$^+$		Infection with P4 *vir1*	
		Cells yielding phage (%)	No. of phage/infected cell	Cells yielding phage (%)	No. of phage/infected cell
C-5204/pUC19		32	15	98	107
C-5204/pGM283	*orf106*	4.5	0.5	18	2.2
C-5204/pFHE1	*orf106*	27	1.6	60	8.4
C-5204/pFHE2A	*orf106am1*	28	7	71	65
C-5204/pGM276	*orf106*, α, *crr*	36	8	100	123

[a] The experiment was performed as described in Materials and Methods. Data are averages of two experiments.

early times when P4 DNA replication is already inhibited, α is actively transcribed. At 60 min, P4 transcripts were almost absent, probably as a consequence of the low copy number of P4 genomes present in the cells.

DISCUSSION

P4 is a natural phasmid (phage-plasmid) that can be propagated both as a temperate phage and as a multicopy plasmid. Autonomous P4 replication, which depends on its α gene product, is regulated: immediately after infection of a sensitive cell, a burst of P4 DNA replication occurs, followed by a decline of DNA copy number; in the plasmid state, P4 copy number is set at about 40 genomes per bacterial chromosome (1). The different replication levels have been correlated to the differential expression of the α gene product. The α gene is more abundantly expressed in the cells shortly after P4 infection than at late times or in P4 plasmid-carrying cells (1, 2, 10). Regulation of α gene expression is achieved both by termination of transcription from promoter P_{LE} and by switching the transcription control of the α operon transcription from P_{LE} to P_{LL} (7, 8). In this work, we identify a new gene (*orf106*) that participates in the control of P4 replication. Gene *orf106* is located immediately upstream of α, and the two genes are transcriptionally coregulated (7, 9). Preliminary analysis using *orf106am* mutants was complicated by the strong polar effect on α expression, which led to a defect in P4 DNA replication; this reduced P4 lytic growth and impaired P4 plasmid propagation. However, analysis of an in-frame deletion in *orf106* made it clear that the lack of Orf106 causes P4 overreplication. Moreover, overexpression of *orf106* from a plasmid inhibits P4 DNA replication, which suggests that *orf106* sets the proper level of P4 DNA replication via a negative regulatory mechanism. The lytic cycle is not significantly affected by the lack of Orf106, but the negative control of P4 replication by this gene product appears to be necessary for the maintenance of the plasmid state. This is probably due to the fact that P4 overreplication occurring in the absence of functional Orf106 leads to host cell death, which prevents stable P4 plasmid propagation. Thus, *orf106* is the first identified P4 gene that is specifically required for plasmid maintenance, and its function appears to be essential for the control of P4 genome replication. We will name this new gene *cnr* (copy number regulation).

Inhibition of P4 replication due to *cnr* expression from a plasmid is not observed if both *cnr* and α are produced. Thus, interference with P4 replication, caused by an excess of Cnr, may be suppressed by increasing the amount of the α gene product. pFHE1, which contains *cnr* and the 5′-terminal half of the α gene, caused a less severe inhibition of P4 growth than pGM283, which carries only *cnr*. Strack et al. (25) found that the same 5′-terminal half of the α gene produced a truncated polypeptide that maintained a low level of primase activity. We suggest that the truncated α protein expressed by pFHE1 may interfere with the inhibitory effect of Cnr.

These data indicate that a balanced ratio of *cnr* to α gene products is essential for proper P4 DNA replication and suggest that Cnr may act on the α gene product activity rather than on the regulation of α gene expression. This view is further supported by the observation that *cnr* does not have any effect on transcription control of the α operon. Our data cannot rule out a negative control of Cnr on α gene translation;

TABLE 5. Effect of *orf106* on P4 *vir1* maintenance in the plasmid state

Bacterial strain[a]	P4 gene(s) carried by the plasmid	Surviving cells[b] (%)	pP4 carriers/ surviving clones (%)	Cured clones (%)
C-2421/pUC19		97	100	<1
C-2421/pGM283	*orf106*	80	<1	100
C-2421/pGM276	*orf106*, α, *crr*	42	66[c]	33

[a] The inducer IPTG (40 μg/ml) was added to the culture 20 min before infection.

[b] The titer of the surviving cells was assayed, in the absence of IPTG, 10 min after infection.

[c] Unstable, segregated cured cells.

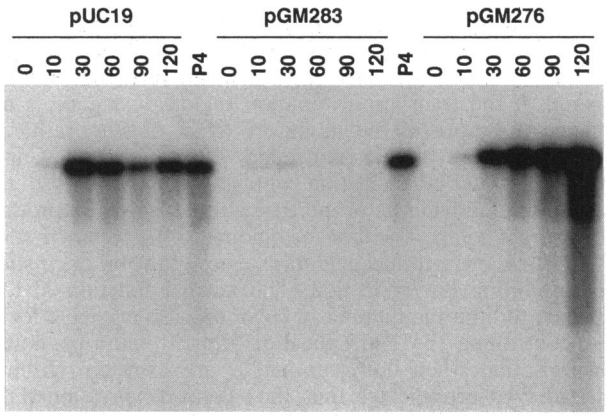

FIG. 5. Southern blot analysis of P4 DNA following infection. C-1a/pUC19, C-1a/pGM283, and C-1a/pGM276 were infected with P4 *vir1*, and total DNA was extracted at different times, digested with *Pvu*II, which linearizes P4, separated by gel electrophoresis, transferred to nitrocellulose filters, and hybridized with a P_{LE}-t2 riboprobe as indicated in Materials and Methods. The plasmids carried by the infected cells and the time points (in minutes) of sampling for DNA extraction are indicated at the top. The lanes marked 0 carry DNA from uninfected C-1a/pUC19, C-1a/pGM283, and C-1a/pGM276, respectively; linearized P4 DNA was loaded in the lanes marked P4.

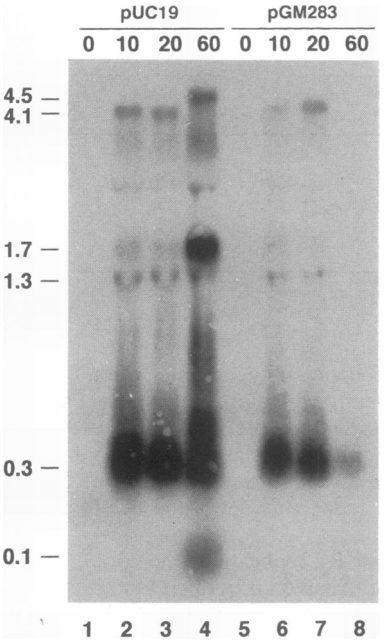

FIG. 6. Effect of *orf106* overexpression on transcription of the α operon, determined by Northern blot of RNA extracted from P4 *vir1*-infected C-2421/pUC19 or C-2421/pGM283 at the times (in minutes) indicated at the top. The RNAs were hybridized to the probe P_{LE}-t2 (P4 coordinates 8418 to 8774). Time zero indicates the uninfected cultures. The experiment was performed as described in Materials and Methods. IPTG (40 μg/ml) was added to the cultures 20 min before the infection.

however, this seems unlikely for the following reasons: (i) there is a good correlation between α gene transcription and amount of α protein present in the cell (1, 2, 7); (ii) when *cnr* and α are coexpressed by a multicopy plasmid, replication of an infecting P4 is enhanced, suggesting that a replication-proficient α gene product is available to the infecting phage; and (iii) full suppression of the inhibitory effect of Cnr is obtained with pGM276, which carries *cnr* and a complete α gene, but not with pFHE1, which contains *cnr* and the 5′-terminal half of the α gene. This observation suggests that the presence on the plasmid of the translation initiation region of α gene is not sufficient to suppress the inhibitory effect of Cnr; rather, a complete α gene product is needed. We suggest that Cnr is directly involved in replication control. Cnr might bind to the α protein, modifying its properties; alternatively, the product of *cnr* might compete with α for binding to the *ori* or *crr* sites. When Cnr is overproduced, it may sequester the *ori* or *crr* sites, thus inhibiting P4 replication. The normal function of Cnr might be to limit the number of *crr* or *ori* sites available for α, thus controlling the P4 replication rate. It must be noted, however, that when both *cnr* and α are expressed from a plasmid, P4 overreplicates; thus, the balanced expression of the genes is not sufficient for controlling P4 copy number, indicating that transcriptional regulation of *cnr* and α is also important for P4. The analysis of the effects of the *cnr* protein on P4 DNA replication in vitro will help elucidate the function of *Cnr*.

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