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Mutants of Plasmid Prophage P1 with Elevated Copy Number: Isolation and Characterization

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A series of mutations of the P1 plasmid prophage that lead to increased copy number was isolated and analyzed. The copy number of the mutants was elevated at least five- to eightfold relative to wild-type P1, as determined by single-cell resistance to antibiotics, activity of enzymes, content of superhelical DNA, and reassociation kinetics. The copy number of two of the mutants was temperature dependent. Based on dominance tests, the mutants fell into two classes, *cis* specific and recessive. The latter class included a temperature-sensitive copy mutant. The existence of a class of recessive mutants suggests that the replication of the P1 plasmid is negatively regulated.

Because of their small size and dispensable nature, plasmids are ideal systems for studying control of DNA replication. The plasmid we have chosen is the prophage P1, which is unusual in that it is the prophage state of a bacteriophage. When present as a plasmid, P1 exhibits properties similar to those of the conjugative R and F plasmids. These include autonomous replication (8), maintenance at a low number of copies per cell (8), a segregation pattern that rarely results in loss from a lysogenic cell (22; J. R. Scott, unpublished data), and integrative suppression of *dnaA* mutants of *Escherichia coli* (2, 4, 16).

Since it has the alternative of replicating in a lytic cycle and of forming infectious phage, P1 has unique advantages for studies of genetic control functions of plasmids. Genetic mapping of most plasmids is time consuming and difficult and usually relies on deletion analysis. Genetic mapping of P1, on the other hand, is relatively easy because it can be done by lytic phage crosses and because a map with many markers that can be scored by spot tests is currently available (32). The existence of a lytic cycle that results in the release of a burst of several hundred progeny allows the simple analysis of a very large number of recombination events from a single cross; a similar simple technique is not available for other plasmids. Deletion mapping can also be performed with P1, usually by the use of spot tests (3, 23, 32). Defective prophage containing deletions and restriction nuclease-generated fragments of P1 DNA cloned into various vectors are currently available for deletion mapping.

A genetic approach to the study of control of DNA replication involves the isolation of mutations which affect plasmid copy number. Such mutations may be in either the host or the plasmid. To test these mutations for complementation, dominance, and effect on incompatibility, cells carrying two differently marked derivatives of the same plasmid (heteroplasmid cells) must be constructed. In the case of plasmids such as R1 or ColE1, conjugation or transformation followed by selection must be used to form heteroplasmid cells. Since selection must be used, early events cannot be studied in these systems. However, P1 can be introduced into P1-carrying strains by infection, so these early events can be readily followed (4a). Because incompatibility between two wild-type P1 plasmids is strong enough to preclude isolation of colonies carrying both (4a), and we did not know whether P1 copy mutants would show less incompatibility, we used another member of the Y incompatibility group, prophage P7, for our dominance test experiments. P1 and P7 share 90% homology (33) and are mutually incompatible, but incompatibility between P1 and P7 is much less strong than between two P1s or two P7s (4a). This reduced incompatibility allows determination of dominance relationships.

In this paper, we describe the isolation, genetic mapping, copy number determination, and dominance relationships of a series of P1 mutants altered in copy control.

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MATERIALS AND METHODS

Media. LB, LB agar, and tryptone are described by Scott (24) and Cowan and Scott (4a). Reference standard sodium ampicillin (Ap) was obtained from Bristol Laboratories, and chloramphenicol (Cm) was obtained from Sigma Chemical Co. For low-level drug resistance, each antibiotic was used at 20 μg/ml in LB. Ampicillin plates were stored in the cold and used within 1 week. For dominance tests, LB plates were supplemented with citrate (4×10^{-3} M) to reduce reinfection by free phage that might be released on the plate. Incubation with high concentrations of antibiotics proceeded for 2 days.

Bacteria. *E. coli* K-12:K140 is streptomycin sensitive and contains no amber suppressors. N99 is derived from W3350, is streptomycin resistant, and contains no amber suppressors. N100 is a *recA* derivative of N99. K175 contains Su-1. W3110 Su^0 and Su-1 are from C. Yanofsky.

Bacteriophage. P1 Ap Cm is a hybrid phage made by crossing P1 Cm (11) with P1 Ap (33). Analysis with restriction endonucleases (C. A. Watkins and J. R. Scott, manuscript in preparation) shows that it consists mostly of P1 DNA, except for the region left of P1 *Bam*HI-4 (where the Ap^r transposon Tn*902* is located) and the substitution of P7 DNA for P1 *Bam*HI-10 and -12 (see reference 1 for restriction map of P1; 4a). The Cm marker (Tn*9*) was spontaneously lost and gave rise to the P1 Ap derivatives we used. P1 Cm *cop*N20 is a recombinant from a cross of P1 Ap Cm *cop*N20 × P1 *am*10.1.

Nitrous acid mutagenesis. Five milliliters of an overnight culture of K140(P1 Ap Cm) was centrifuged and suspended to the original volume in 0.05 M acetate buffer (pH 4.6). These cells were centrifuged and suspended in 0.3 ml of sodium nitrite dissolved in acetate buffer (pH 4.6; final concentration, 0.05 M) and incubated for 10 min at 37°C. Then 4.7 ml of LB was added, and the cells were centrifuged and resuspended in LB, distributed into separate culture tubes, and left to grow overnight at 37°C. As a control to be sure the mutants in each tube were of independent origin, a sample was plated on selective medium before overnight incubation. As a control for spontaneous mutation, a culture that had not been incubated with nitrous acid was similarly treated. The selective conditions were either tryptone plates containing 1 mg of both ampicillin and chloramphenicol per ml incubated at 42°C (which led to isolation of *cop*N3) or LB plates containing 2 mg of ampicillin and 1.5 mg of chloramphenicol per ml incubated at 37°C (which led to isolation of all other mutants with "N" prefix).

To recover the mutant phage, overnight cultures of each mutant were grown with 2.5×10^{-2} M $CaCl_2$, sterilized with chloroform, and centrifuged to remove bacteria, and the supernatants were plated for phage on an Su^+ and an Su^0 host. Plaques were picked and spotted directly onto LB containing ampicillin (1 mg/ml). As a control, plaques of the P1 Ap Cm parent were used. Phage were purified by streaking single plaques at least twice on indicator bacteria, and lysates were grown from single plaques.

Ethyl methane sulfonate mutagenesis. A log-phase culture of K140(P1 Ap Cm) was concentrated twofold into 0.2 M Tris buffer (pH 7.5), and 60 μl of ethyl methane sulfonate was added. This culture was shaken at 37°C for 2 h, the cells were diluted both 10- and 100-fold to LB, and equal portions were separated into individual tubes and incubated overnight at 40°C. The next day, samples of each were spread on LB plates containing 1 mg of both ampicillin and chloramphenicol per ml, and the plates were incubated at 40°C. The titer of drug-resistant cells in each treated sample exceeded that in the nonmutagenized control by more than 10-fold. To screen for temperature-sensitive *cop* mutants, colonies were picked and streaked again on the same selective medium at both 30 and 40°C. After purification, a log-phase culture of the apparent mutants was assayed at both temperatures with and without 1-mg/ml concentrations of both antibiotics in the plates. Mutant lysogens plating with 100% efficiency in the presence of drugs were saved.

Assay for β-lactamase. The preparation of the crude cell extract is from Lindqvist and Nordstrom (13), and the enzyme assay is adapted from Imsande (9). Cells were grown to mid-log phase (5×10^8/ml) and concentrated 10-fold into Tris buffer (0.03 M, pH 8.0) containing 20% sucrose. Lysozyme and EDTA were used to remove the cell wall (56 μl of 1-mg/ml lysozyme plus 56 μl of 0.1 M EDTA at pH 8.0), and the mixture was incubated 30 min at room temperature. $CaCl_2$ was added to give a concentration of 0.01 M, and the cells were removed by centrifugation in the cold. The supernatant contained the crude preparation used for assays.

A 2.4-ml portion of cacodylate-bromothymol blue mixture (0.003 M sodium cacodylate; 4 mg of bromothymol blue per 100 ml, pH 7.3) mixed with 0.44 ml of water was the "assay mixture." A 60-μl amount of a 100-mg/ml solution of sodium ampicillin was added to the cuvette, followed by addition to the assay mixture of 0.1 ml of enzyme extract. The samples were mixed, and the decrease in optical density was read at 620 nm. A blank containing no enzyme extract was included with each sample.

Assay for chloramphenicol acetylase. The chloramphenicol acetylase assay was adapted from Davis and Vapnek (5). Log-phase cells were collected by centrifugation, washed with 0.1 M Tris buffer (pH 7.8), and collected as pellets. Cells were lysed by a 5-min agitation on a Vortex mixer in 1 ml of 0.5% Sarkosyl, 1% toluene, and 0.01 M Tris (pH 7.8). The crude preparations were centrifuged to remove cell debris and used for the enzyme assay.

The reaction mixture contained 0.1 ml of 5,5'-dithiobisnitrobenzoic acid solution (4 mg/ml in 70% ethanol), 0.1 ml of 0.1 M Tris (pH 7.8), 0.1 ml of chloramphenicol solution (0.01 M in 70% ethanol), and water to 1 ml. This was mixed thoroughly, and 10 to 15 μl of enzyme extract was added. After further mixing, 20 μl of 0.01 M acetyl coenzyme A was added to start the reaction, and the optical density of 412 nm was recorded. The blank contained no chloramphenicol.

Procedure for protein concentration. To determine protein concentration, we used a procedure adapted from Lowry et al. (14; S. Kushner, personal communication). The crude enzyme preparations were diluted appropriately, and 0.1 ml was added to 0.4 ml of water and 0.5 ml of standard reagent composed of 48 parts of A:1 part of B:1 part of C (A is 3% Na_2CO_3 in 0.1 N NaOH; B is 3.27% sodium tartrate in water; C is 2% $CuSO_4 \cdot 5H_2O$). Then 0.1 ml of half-strength phenol reagent (Folin and Ciocalteu phenol reagent; Sigma

Chemical Co.) was added, and the mixture was incubated for 30 min at room temperature. The protein concentration was determined by comparison of the optical density at 750 nm with that of dilutions of bovine serum albumin (Sigma).

Determination of P1 plasmid copy number by equilibrium centrifugation in CsCl-ethidium bromide. Log-phase cultures were labeled with [^{3}H]thymidine followed by cell lysis and equilibrium centrifugation in CsCl-ethidium bromide gradients (31). The amount of P1 plasmid DNA present as covalently closed circular (CCC) molecules was determined from the ratio of counts in the two peaks.

Determination of P1 plasmid copy number by DNA-DNA hybridization. Isolation of the DNA used in the DNA-DNA hybridization experiments is described by Chesney et al. (2). Preparation of radio-labeled P1 Ap Cm plasmid DNA was performed by nick translation as described by Shepard and Polisky (26). Specific activity was about 2×10^7 dpm/μg. DNA hybridizations were performed by using an S1 endonuclease assay (20), which is a modification of the technique of Maxwell et al. (15). The hybridization reaction mixtures contained 25 μl of 1 M Tris-hydrochloride (pH 8.0), 25 μl of calf thymus DNA (1 mg/ml), which was used to increase the S1 endonuclease-degrading activity, excess cold driver DNA [approximately 6 μg for N99 and N99(P1 Ap Cm) and 1 to 2 μg for each of the P1 *cop* mutants], and water in a final volume of 225 μl. Hybridization reactions were performed in 3-ml Pyrex glass conical tubes containing a small amount of mineral oil to prevent evaporation. The reaction mixtures were boiled for 5 min to denature the DNA and then placed on ice for 2 min to prevent spontaneous renaturation. The hybridization process was begun by adding 25 μl of 5 M NaCl to each tube (final concentration, 0.5 M) and placing the tubes in a 68°C water bath. At intervals of 0, 5, 10, 20, 30, 60, and 90 min and 24 h, 25-μl samples of the hybridization reaction mixtures were added to Eppendorf tubes on ice containing 225 μl of S1 nuclease buffer (1 mM $ZnSO_4$, 5 mM 2-mercaptoethanol, 0.2 M NaCl, and 25 mM sodium acetate, pH 4.5). After the final 90-min samples were taken, two 50-μl samples for each time point were added to duplicate Whatman DE81 filters. Then 3 μl of S1 endonuclease (Sigma; 2 U/μl) was added to the remainder of the sample in each tube, and the digestion was allowed to proceed at 37°C for 90 min. Duplicate 50-μl samples again were spotted onto DE81 filters after S1 digestion was completed. The filters were washed three times for 5 min each in 0.48 M sodium phosphate buffer (pH 6.8) to remove S1 digestion products. The progress of renaturation at each time point was determined by dividing the counts per minute after S1 digestion by the counts per minute before S1 digestion. The observed rate constants for the reassociation of ^{32}P-labeled P1 DNA in the presence of various DNA samples were calculated by fitting the experimental points to the appropriate curve, using a nonlinear least-squares regression computer program developed by Britten and Davidson as described in reference 6. The DNA-DNA renaturation reaction is described by the expression $C = C_0/(1 + k_2 C_0 t)^{0.44}$, where C is the transient single-stranded DNA concentration at time t, k_2 is the rate constant for the renaturation reaction, and C_0 is the initial single-stranded DNA concentration. The pure rate constant for renaturation of ^{32}P-labeled P1 DNA was determined by hybridizing 0.02 μg of ^{32}P-labeled P1 DNA to 0.2, 0.4, and 0.6 μg of purified P1 Ap Cm DNA and averaging the resulting three rate constants as determined by the computer program. P1 copy numbers were determined as described by Gelb et al. (7).

The molecular weight of P1 Ap Cm plasmid DNA is 69×10^6 (Watkins and Scott, manuscript in preparation). The molecular weight of *E. coli* DNA per chromosome is 2.7×10^9 (12). The molecular weight of *E. coli* DNA/molecular weight of P1 DNA is 39. ^{32}P-labeled P1 copies/*E. coli* chromosome is expressed as micrograms of ^{32}P-labeled P1 DNA/microgram of total cell DNA × 39. Factor of increased rate is (observed k_2 of ^{32}P-labeled P1 DNA plus total cell DNA)/(pure k_2 for ^{32}P-labeled P1 DNA). Total concentration of P1 DNA is (^{32}P-labeled P1 molecules/*E. coli* chromosome) × factor of increased rate. P1 copies per chromosome equals total concentration of P1 DNA − (^{32}P-labeled P1 copies/*E. coli* chromosome).

Infection for phage cross and dominance test. N100(P7 Cm) was grown to 10^8 cells/ml in LB, concentrated threefold to Tris buffer (0.01 M, pH 7.6), and infected with a multiplicity of infection of five phage per cell. $CaCl_2$ was added to a final concentration of 5×10^{-2} M, and adsorption was allowed to proceed for 10 min at 37°C. Unadsorbed phage was removed by centrifugation.

Phage crosses. Infected cells were diluted at least 10-fold into LB containing 2.5×10^{-2} M $CaCl_2$ and incubated at 37°C with aeration for 90 min. The mixture was sterilized with chloroform. Total phage was assayed on K175 and *am*$^+$ recombinants scored on K140(λ). Recombinant plaques were picked with sterile applicator sticks and spotted onto LB plates with a soft agar overlay containing N99(λ) and 10 mg of streptomycin (to kill the K140 cells and any K140 lysogens containing parental *am* phage that may have been transferred). The spots were incubated overnight to form N99(P1 *am*$^+$) lysogens. The next day, applicator sticks were used to transfer samples from each spot directly onto LB plates containing 2.8 mg of ampicillin per ml. Appropriate controls were included on each plate. Cop$^+$ phage do not produce spots of bacterial growth at this ampicillin concentration, whereas Cop$^-$ phage do.

Dominance test. Infected cells were diluted 10-fold into 37°C prewarmed LB with 4×10^{-3} M citrate. The culture was aerated and, at various time intervals, assayed on appropriate media.

RESULTS

Mutant isolation. To isolate plasmid mutants maintained at a higher copy number than the wild type, we used antibiotic selection. Lysogens for P1 Ap Cm, in which the two resistance markers are separated by about one-third of the P1 genome length (see Fig. 1), were mutagenized. Selection for high-level resistance to both antibiotics was used to prevent isolation of variants in which the antibiotic resistance gene had been amplified. To permit isolation of both amber and temperature-sensitive copy number mutants (*cop*), the mutagenized lysogen contained no nonsense suppressor, and selection was per-

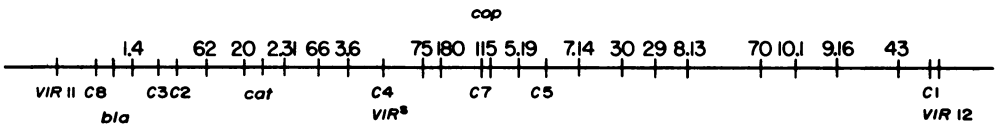

FIG. 1. Map of P1 Ap Cm showing *am* mutants above the line and clear (*c*) and virulent (*vir*) markers below (adapted from Razza et al. [21]). It is not drawn to scale. *cat* denotes the gene for chloramphenicol acetyltransferase, and *bla* denotes the gene for β-lactamase. The location of *cop* is indicated above the map.

formed at 40°C. Only one mutant was retained from each mutagenized sample to assure that each was of independent origin. The first mutants (designated "N" followed by a number) were obtained after treatment with nitrous acid, but their frequency did not exceed the spontaneous frequency of *cop* mutations. The last mutants were isolated after mutagenesis with ethyl methane sulfonate and designated "E" followed by a number.

In this study, we were interested only in single gene or site mutations in the phage. To show that the mutation was carried by the phage and that it was not due to gross alterations of the DNA, plaque-forming phage were isolated from the selected lysogen and tested to be sure that when they lysogenized a new host, they converted it to high-level ampicillin and chloramphenicol resistance. P1 virions are produced by packaging a "headful" of DNA and therefore are unable to contain a large addition to the original wild-type amount of DNA; large deletions would result in loss of genes essential for lytic growth. Therefore, the isolation of "converting" plaque-forming phage indicated that the mutations were not caused by gross DNA alterations. So far, all phage-producing, highly resistant derivatives of P1 Ap Cm lysogens carry the mutation in the phage DNA; i.e., no host mutations affecting P1 copy number have been isolated by this procedure. Not all of the colonies that grew on high-ampicillin and -chloramphenicol plates produced plaque-forming phage, however; those that did not produce phage contained large deletions in the plasmid DNA and were designated "Δ*cop*" (Watkins and Scott, manuscript in preparation).

Relative copy number by enzyme assay. The specific activity of the enzyme coded by each antibiotic resistance determinant of the mutant plasmid was compared with the activity of the enzyme in the wild-type parent. Since each plasmid contained two antibiotic resistance determinants, Cm and Ap, the relative enzyme activity (mutant/wild type) of chloramphenicol acetyltransferase should serve as an internal control for the relative activity of β-lactamase. However, the relative copy number determined by chloramphenicol acetyltransferase activity was higher than that based on β-lactamase activity. This discrepancy might have been caused by the lack of reproducibility in the data, presumably because crude enzyme extracts were assayed. Accuracy is especially important in determining the activity of the wild type, since copy number is expressed relative to this. Table 1 shows the poor reproducibility in determination of enzyme activities of the wild type. From the β-lactamase activity assays, it appeared that the relative copy number of most of the mutants was 12 to 16, and the chloramphenicol acetyltransferase assays indicated the copy number of the mutants to be 18 to 21 times that of the wild type.

Relative copy number by single-cell resistance. To determine single-cell resistance levels, exponentially growing cultures of mutant and wild-type lysogens were diluted, and about 200 cells were spread per plate. The plates contained graded increases in ampicillin or chloramphenicol concentration. After incubation, the colonies were counted, and the highest concentration of antibiotic that gave an efficiency of plating (EOP) of 100% (relative to that on drug-free plates) was determined. In the N99 lysogen series, the chloramphenicol resistance level indicated a relative copy number of four to five for *cop*N3, -N20, -N22, and -N24 and about three for *cop*N26 (Fig. 2; Table 2). N99(P1 Ap Cm *cop*N26) is unstable, so for this mutant, the EOP on high antibiotic concentrations was compared with that at 20 μg/ml instead of that in the absence of antibiotics. For N99 lysogens carrying *cop*N3, -N20, -N22, and -N24, the ampicillin resistance was about 18 to 24 times that of N99 (P1 Ap Cm). Thus, the resistance of mutants relative to the wild type was much higher for ampicillin than for chloramphenicol. At 42°C, N99(P1 Ap Cm *cop*E38) and N99(P1 Ap Cm *cop*E47) were about four times as chloramphenicol resistant as the wild-type lysogen (Table 2).

Relative copy number determined by equilibrium centrifugation. The relative amount of CCC DNA was determined for each lysogen by equilibrium centrifugation of [^{3}H]thymidine-labeled DNA in the presence of CsCl-ethidium bromide (Table 3; Fig 3). Assuming that all plasmid DNA was in the CCC form, in N99, *cop*N3, -N20, -N22, -N24, and -N26 at 32°C and E38 at 40°C had about four to seven times the copy number of P1 Ap Cm. This is close to the copy

TABLE 1. Relative copy number determined by enzyme activity

Strain	β-Lactamase		Chloramphenicol acetyltransferase	
	Sp act (U/mg of protein)[a]	Relative copy no.[b]	Sp act (U/mg of protein)	Relative copy no.
N99(P1 Ap Cm)	0.046 ± 0.012	1	0.76 ± 0.30	1
N99(P1 Ap *cop*N3)	0.68 ± 0.19	15		
N99(P1 Ap Cm *cop*N22)	0.56 ± 0.14	12	16 ± 2.5	21
N99(P1 Ap Cm *cop*N24)	0.58 ± 0.08	13	13 ± 2.2	18
N99(P1 Ap Cm *cop*N26)	0.75 ± 0.39	16	13 ± 2.0	18

[a] The standard deviation of each experimental determination is given.
[b] Calculated as specific activity of the mutant divided by that of the wild type.

number deduced from single-cell resistance data for chloramphenicol (Table 2).

Relative copy number determined by DNA-DNA hybridization. For each P1 *cop* mutant, plasmid copy number per *E. coli* chromosome was determined by analysis of the reassociation kinetics. Table 4 shows the results for two independent sets of experiments. The copy number for P1 Ap Cm was approximately one, which agrees with previous determinations for P1 (8, 17). In N99, all of the P1 *cop* mutants tested had five to eight copies per chromosome, except P1 Ap Cm *cop*E38(Ts), which appeared to have three to five copies at 40°C. In W3110, *cop*E101 and *cop*E205 had about four to six copies.

Map location of *cop* mutations. To locate the *cop* mutations on the P1 genetic map, spontaneously released phage from each lysogen were purified and used in phage crosses. An amber mutation was introduced into each *cop* mutant by a cross, and the *am cop* double mutant was crossed with a series of *am* mutants whose map locations had been previously established (Fig. 1). The results indicated that all of the *cop* mutations were closely linked and that they were located near *am*115 (Table 5). They did not distinguish whether the *cop* mutations were to the right or to the left of this marker.

Restriction pattern of *cop* mutants. To be certain that there had been no major DNA rearrangements in the *cop* mutants, the patterns generated by electrophoresis of DNA fragments

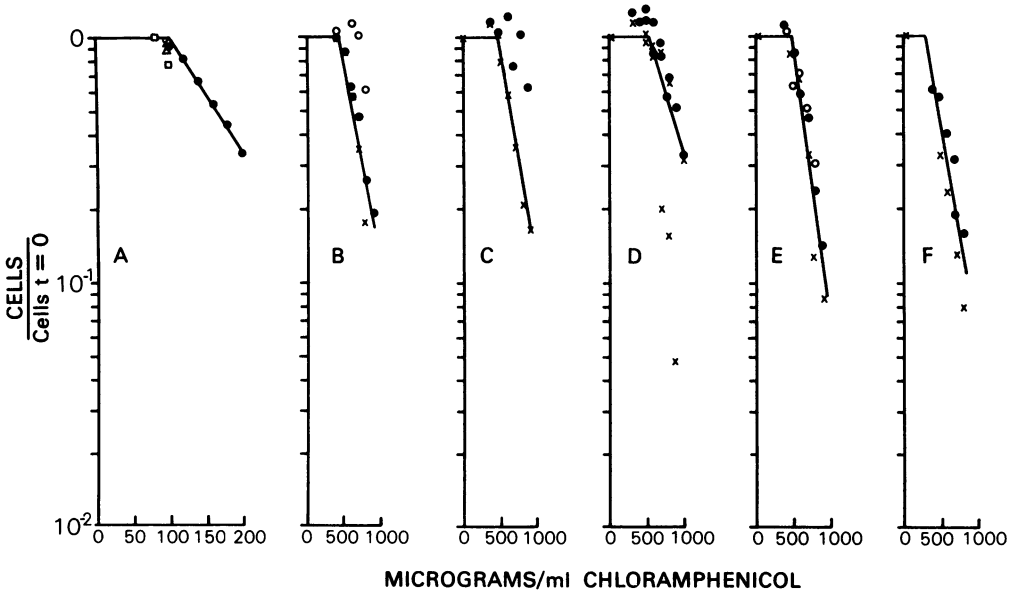

FIG. 2. Resistance to chloramphenicol. Log-phase cultures of N99 lysogens were assayed on LB containing the indicated chloramphenicol concentrations. The plasmids were: A, P1 Ap Cm; B, P1 Cm *cop*N3; C, P1 Ap Cm *cop*N20; D, P1 Ap Cm *cop*N22; E, P1 Ap Cm *cop*N24; and F, P1 Ap Cm *cop*N26. Each symbol indicates results from an independent experiment. The normalized cell titer is the number of colonies on the indicated chloramphenicol concentration divided by the number on medium without chloramphenicol.

TABLE 2. Relative copy number based on antibiotic resistance[a] in N99 lysogens

Lysogen	Ampicillin	Chloramphenicol
*cop*N3	21	4
*cop*N20	24	5
*cop*N22	18	5
*cop*N24	18	5
*cop*N26		3
*cop*E38 (42°C)		4
*cop*E38 (30°C)		1
*cop*E47 (42°C)		4
*cop*E47 (30°C)		1

[a] Relative resistance is the highest antibiotic concentration that gives an EOP of 1 for the mutant, divided by the concentration that gives an EOP of 1 for the wild-type lysogen. The maximum concentration at which N99 (P1 Ap Cm) has an EOP of 1 is 100 μg/ml for chloramphenicol and 80 μg/ml for ampicillin.

TABLE 3. P1 copy number determined by CsCl-ethidium bromide equilibrium centrifugation of [^{3}H]thymidine-labeled DNA

Strain	% CCC DNA[a]	P1 copies/chromosome[b]
N99(P1 Ap Cm)	2.6	1.0
N99(P1 Ap *cop*N3)	11	4.3
N99(P1 Ap Cm *cop*N20)	19	7.4
N99(P1 Ap Cm *cop*N22)	15	5.9
N99(P1 Ap Cm *cop*N24)	12	4.7
N99(P1 Ap Cm *cop*N26)	9.8	3.8
N99(P1 Ap Cm *cop*E38) (Ts) at 40°C	13	5.1
N99(P1 Ap Cm *cop*E38) (Ts) at 30°C	6.0	2.3

[a] The ratio of CCC DNA to chromosomal DNA was determined by dividing the total counts per minute in the CCC fraction of the gradient by the total counts per minute in the chromosomal fraction. Representative gradients are shown in Fig. 3.

[b] The plasmid copy numbers were determined by using 2.56×10^{-2} as the ratio of molecular weight of plasmid/chromosome (see Materials and Methods).

produced by *Bam*HI, *Bgl*II, and *Eco*RI of the parental P1 Ap Cm plasmid DNA were compared with that of *cop*N3, -N20, -N22, and -N26 (data not shown). No differences were detectable, except in the case of P1 Ap *cop*N3, which had lost the Cm marker during phage purification. This is a common IS*1*-promoted type of deletion in phage P1. No miniplasmids were detected in any of the lysogens.

Growth rate of *cop* mutant lysogens. The growth rate of the N99 series of *cop* mutant lysogens was compared with that of the nonlysogenic parent. The presence of the extra copies of the plasmid did not decrease the growth rate of this strain in rich medium (LB), measured by

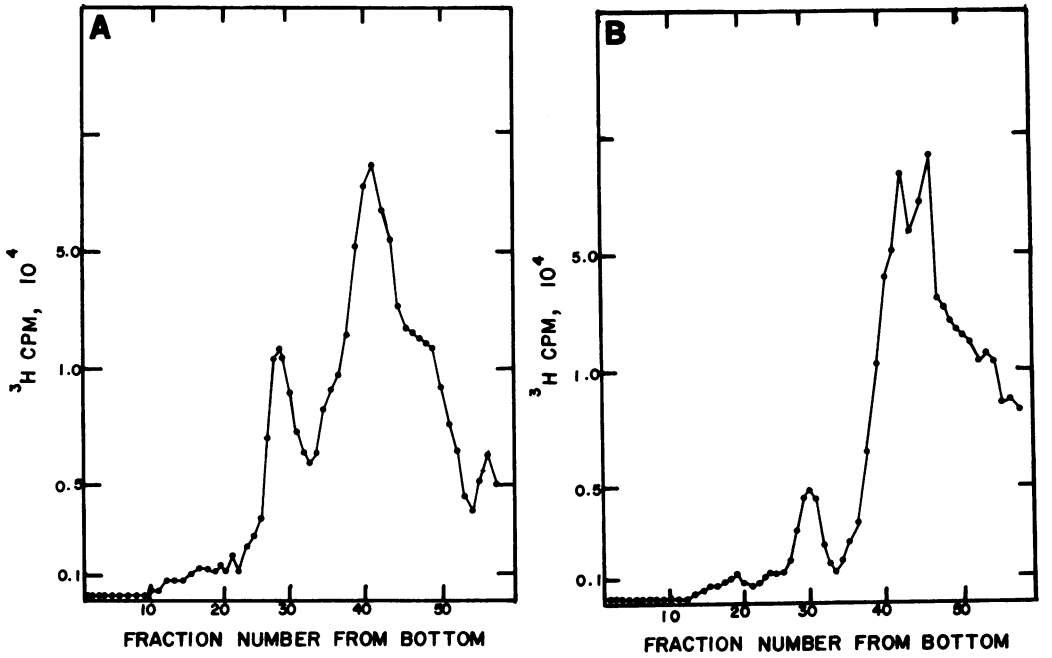

FIG. 3. CsCl-ethidium bromide profiles of DNA. Cells were labeled with [^{3}H]thymidine followed by cell lysis and equilibrium centrifugation. The amount of P1 present as CCC molecules was determined from the ratio of counts in the two peaks. A, N99(P1 Ap Cm); B, N99(P1 Ap Cm *cop*N24).

TABLE 4. Plasmid copy number determined by reassociation kinetics[a]

Origin of DNA driver	Expt no.	μg of driver added	Observed rate constant (k_2)	Computed % plasmid	P1 copies/ *E. coli* chromosome	Relative copy no.
N99(P1 Ap Cm)	1	7.1	491	2.2	0.86	1.0
	2	6.3	808	2.3	0.88	1.0
N99(P1 Ap Cm *cop*N20)	1	1.6	578	12	4.8	5.6
	2	1.2	1,051	16	6.2	7.0
N99(P1 Ap Cm *cop*N22)	1	1.5	663	15.6	6.1	7.1
	2	1.1	905	15	5.8	6.6
N99(P1 Ap Cm *cop*N24)	1	1.6	817	19	7.4	8.6
	2	1.3	1,206	17	6.7	7.6
N99(P1 Ap Cm *cop*N26)	1	1.7	643	13	5.1	5.9
	2	1.5	1,382	17	6.8	7.7
N99(P1 Ap *cop*N3)	1	1.5	630	14.6	5.7	6.6
	2	1.6	953	11	4.2	4.8
N99(P1 Ap Cm *cop*E38) (Ts) at 40°C	1	1.7	390	6.6	2.6	3.0
	2	1.4	826	10.5	4.1	4.7
N99(P1 Ap Cm *cop*E38) (Ts) at 30°C	1	1.1	260	4.9	1.9	2.2
	2	1.3	368	4.1	1.6	1.8
W3110 Su^0 (P1 Ap Cm)		6.1	4,758	3.3	1.3	1.0
W3110 Su^1 (P1 Ap Cm)		5.2	3,219	2.5	1.0	1.0
W3110 Su^0 (P1 Ap *cop*E101)		6.0	20,990	15	5.8	4.5
W3110 Su^1 (P1 Ap *cop*E101)		5.3	17,930	14	5.6	5.6
W3110 Su^0 (P1 Ap *cop*E205)		6.2	19,580	13	6.1	4.7
W3110 Su^1 (P1 Ap *cop*E205)		4.5	14,910	14	5.5	5.5

[a] ^{32}P-labeled P1 DNA was annealed with excess unlabeled total cell DNA at the listed concentrations. The kinetics of renaturation were followed, and the observed second-order rate constants were determined as described in Materials and Methods. Percentage of plasmid DNA and P1 plasmid copies per chromosome were calculated as in Gelb et al. (7). The pure rate constant for the reassociation of ^{32}P-labeled P1 DNA was 140 in experiment 1 and 108 in experiment 2.

viable counts or by microscopic particle counts (data not shown).

Dominance tests. To define the genes involved in control of P1 plasmid replication and to attempt to determine their interaction and function, complementation tests between the wild type and copy mutants were performed. If the mutant is dominant to the wild type, then in cells with both types of plasmids, the copy number of the wild type should be higher than normal. On the other hand, if the wild type is dominant to the mutant, then the copy number of the mutant should drop to the wild-type level in cells with both types of plasmids. If the mutation is *cis* specific, then the number of mutant plasmids will remain high and the number of wild type plasmids will remain low in cells carrying both.

Because incompatibility of two P1 plasmids precludes isolation of colonies carrying both (4a), we used the closely related phage P7 in the dominance tests as the cop^+ source. The frequency of cells carrying two different plasmids was never close to 100% of the population (Table 6, column 1), so physical methods could not be used to determine the copy number of each plasmid in cells containing both. Instead, the degree of resistance to antibiotics was used as a measure of copy number. Each plasmid was marked with a different antibiotic resistance, and cells carrying both were selected by plating on medium containing both drugs. The level of chloramphenicol resistance of a P1 *cop* lysogen was proportional to the plasmid copy number as determined by DNA hybridization, and the level of ampicillin resistance of the mutants was higher than expected from the relative copy number (see above). Using either resistance marker, it was therefore easy to distinguish between the wild-type copy number and an elevated one.

For the dominance test, N100(P7 Cm) was infected at a multiplicity of 5 with each P1 Ap *cop* mutant in turn. At several different times after infection, the cells were assayed on a series of plates, all of which contained at least 20 μg of both ampicillin and chloramphenicol per ml. In one series the concentration of ampicillin was increased, and in another the chloramphenicol concentration went up. As a control to identify recombinant P1 Ap Cm *cop* plasmids, cells were assayed on plates with high concentrations of both antibiotics. The highest concentration used (500 μg of ampicillin and 400 μg of chloramphenicol per ml) was one which gave efficient plating of N100(P1 *cop*) (Table 7).

To determine the time it took for a P1 Ap *cop* phage to infect, establish a plasmid with high

TABLE 5. Map location of *cop* mutants

Phage crossed	Total am^+ plaques scored	% *cop*
*cop*N3 *am*5.19 × *am*3.6	387	51.9
× *am*180	388	64.4
× *am*115	385	68.6
× *am*8.13	968	22.7
× *am*7.14	388	36.1
Relative distance from *cop*N3: (115, 180), (5.19, 3.6), 7.14, 8.13		
*cop*N3 *am*9.16 × *am*2.31	436	66.0
× *am*3.6	581	72.6
× *am*180	388	83.0
× *am*115	184	87.5
× *am*7.14	248	83.0
× *am*8.13	284	69.0
× *am*43	200	50.0
Relative distance from *cop*N3: (115, 180, 7.14), 3.6, (8.13, 2.31), (9.16, 43)		
*cop*N20 *am*5.19 × *am*3.6	732	51.8
× *am*180	288	66.3
× *am*115	288	81.9
× *cop*N3 *am*9.16	1295	99.7
Relative distance from *cop*N20: *cop*N3, 115, 180, (5.19, 3.6)		
*cop*N22 *am*5.19 × *am*3.6	485	47.4
× *am*180	291	63.6
× *am*115	291	75.9
× *cop*N3 *am*9.16	340	99.7
Relative distance from *cop*N22: *cop*N3, 115, 180, (5.19, 3.6)		
*cop*N24 *am*5.19 × *cop*N3 *am*9.16	230	72.6
× *am*180	383	62.7
× *am*115	783	71.0
Relative distance from *cop*N24: (*cop*N3, 115, 180), 5.19		
*cop*N26 *am*5.19 × *am*3.6	383	50.9
× *am*180	408	68.4
× *am*115	377	74.8
× *cop*N3 *am*9.16	429	98.4
Relative distance from *cop*N26: *cop*N3, 115, 180, (5.19, 3.6)		
*cop*E38 *am*5.19 × *am*3.6	550	60.0
× *am*180	400	71.0
× *am*115	400	77.8
× *cop*N3 *am*9.16	499	99.6
Relative distance from *cop*E38: *cop*N3, (115, 180), 3.6, 5.19		

copy number, and express its resistance to high ampicillin concentration, N100 was infected with each P1 Ap *cop* mutant in turn, and the infected culture was assayed at different times on a series of plates with increasing ampicillin concentration. For all mutants used, by 2.5 h, resistance to 500 μg of ampicillin per ml was expressed by at least 90% of the plasmid-containing cells (defined by resistance to 20 μg of ampicillin per ml) (data not shown). There is no good control to determine the length of time it takes for a resident Cm plasmid to increase its copy number and express high resistance, so assays for high drug resistance were performed at 3.5 h.

After infection of N100(P7 Cm) by *cop*N3, -N22, and -N24, high ampicillin resistance was expressed (compare with N100(P1 Ap Cm) (Table 7), whereas chloramphenicol resistance remained low. This means that neither the mutant nor the wild type was dominant; these mutants were *cis* specific. In the case of P1 Ap *cop*N20, the frequency of recombinants (resistant to 400 μg of both ampicillin and chloramphenicol per ml) was high, and this made it impossible to determine whether *cop*N20 was dominant or recessive to the wild type. Electrophoresis of plasmid DNA from single colonies of N100(P7 Cm) infected with P1 Ap *cop*N20 from the plates with high concentrations of both drugs indicated that these Ap Cm *cop*N20 recombinants had about twice the molecular weight of P1 Ap Cm (T. Sterling and J. R. Scott, unpublished data). We constructed P1 Cm *cop*N20 by crossing out the Ap marker and used it to infect N100(P7 Ap). The results of this infection indicated that *cop*N20 was also *cis* specific (data not shown).

In contrast, with *cop*E38, *cop*E47, *cop*E205 and *cop*E101, the incoming copy mutant did not express resistance to high ampicillin concentrations in the presence of P7 Cm (Table 6). This indicated that the wild type prevented the high copy number of the incoming mutant from being expressed. In a control, when each of these mutants infected nonlysogenic N100, at least 90% of the lysogens were resistant to 500 μg of ampicillin per ml by 3.5 h after infection (data not shown). These mutants, therefore, appeared to be recessive to the wild type.

DISCUSSION

The survivors of a P1 Ap Cm lysogen resistant to high concentrations of ampicillin and chloramphenicol fall into two classes: those which release nondefective phage that transmit the high-copy-number phenotype by lysogenization of a new host and those which produce no viable phage because their prophage contains internal duplications and deletions. Only the former were included in this study.

The purified phage spontaneously released from each independent mutant isolate was used to lysogenize an antibiotic-sensitive strain, and the copy number of the plasmid prophage in the new lysogens was determined by four different

TABLE 6. Infection of *recA*(P7 Cm) with P1 Ap *cop* mutants

Infecting phage	Ap^r Cm^r/total viable cells at 15 min	EOP relative to Ap^{20} Cm^{20}[a]							
		Ap^{200} Cm^{20}	Ap^{300} Cm^{20}	Ap^{400} Cm^{20}	Ap^{500} Cm^{20}	Ap^{400} Cm^{400}	Ap^{20} Cm^{200}	Ap^{20} Cm^{300}	Ap^{20} Cm^{400}
P1 Ap *cop*N3	2.9×10^{-3}	4.89×10^{-1}	2.6×10^{-1}	1.6×10^{-1}	4.72×10^{-2}	$<10^{-4}$	3.7×10^{-2}	8.7×10^{-4}	1×10^{-4}
P1 Ap *cop*N22	4.92×10^{-3}	5.84×10^{-1}	2.57×10^{-1}	2.99×10^{-1}	4.42×10^{-2}	$<2.10^{-3}$	4.65×10^{-2}	$<2 \times 10^{-3}$	$<2 \times 10^{-3}$
P1 Ap *cop*N24	1.2×10^{-1}	4.5×10^{-1}	3.7×10^{-1}	1.0×10^{-1}	2.3×10^{-2}	2×10^{-5}	3.1×10^{-2}	1.8×10^{-3}	4.2×10^{-4}
P1 Ap *cop*N20	2.06×10^{-3}	4.55×10^{-1}	3.98×10^{-1}	2.27×10^{-1}	1.55×10^{-1}	7.95×10^{-2}	1.31×10^{-1}	9.66×10^{-2}	5.68×10^{-2}
P1 Ap *cop*E101	1.6×10^{-2}	9.7×10^{-2}	$<4 \times 10^{-4}$	$<4 \times 10^{-4}$	$<4 \times 10^{-4}$	$<2 \times 10^{-4}$	4×10^{-4}	$<2 \times 10^{-4}$	$<2 \times 10^{-4}$
P1 Ap *cop*E38[b]	9.5×10^{-4}	1.6×10^{-2}	3×10^{-3}	$<1 \times 10^{-3}$	$<1 \times 10^{-3}$	$<1 \times 10^{-3}$	1×10^{-3}	$<1 \times 10^{-3}$	$<1 \times 10^{-3}$

[a] The superscripts refer to the antibiotic concentration (micrograms per milliliter) in the medium.
[b] At 40 and 42°C.

TABLE 7. Resistance of P1 *cop* and *cop*$^+$ lysogens[a]

N100 lysogen	EOP[b]							
	Ap^{200} Cm^{20}	Ap^{300} Cm^{20}	Ap^{400} Cm^{20}	Ap^{500} Cm^{20}	Ap^{400} Cm^{400}	Ap^{20} Cm^{200}	Ap^{20} Cm^{300}	Ap^{20} Cm^{400}
P1 Ap Cm	5.2×10^{-4}	2.1×10^{-5}	1.6×10^{-5}	1.7×10^{-5}		5.8×10^{-2}	2.6×10^{-3}	6.5×10^{-4}
P1 Ap Cm *cop*N20				10^0	10^0			10^0
P1 Ap Cm *cop*N22				10^0	10^0			10^0
P1 Ap Cm *cop*N24				10^0	8.5×10^{-1}			7.8×10^{-1}
P1 Ap Cm *cop*E101	1.1×10^0	7.6×10^{-1}	7.7×10^{-1}	3.5×10^{-1}	3.2×10^{-1}	6.3×10^{-1}	5.4×10^{-1}	2.2×10^{-1}
P1 Ap Cm*cop*E38[c]	1.0×10^0	8.4×10^{-1}	9.5×10^{-1}	8.1×10^{-1}	9.7×10^{-1}	8.9×10^{-1}	1.0×10^0	1.0×10^0

[a] The wild type, N100(P7 Cm), showed no resistance to ampicillin at 200, 300, 400, or 500 μg/ml and resistance to chloramphenicol as follows: 200 μg/ml, 3.14×10^{-2}; 300 μg/ml, 2.35×10^{-4}; 400 μg/ml, 8.52×10^{-5}.
[b] See Table 6.
[c] At 42°C.

methods. All four methods demonstrated an increase in the copy number of the mutant by a factor of at least 4 to 8 over the wild type. We consider the copy number determination by DNA-DNA hybridization to be the most accurate, since this is the most direct measurement of the number of plasmids per chromosome.

The level of antibiotic resistance of single-plasmid-containing cells is the most rapid assay for copy number. The single-cell resistance method measures the minimum copy number, since if there are some cells in the population with a copy number lower than the average, these will not grow on plates containing as high an antibiotic level as the average cell in the population, and the measured EOP will be less than 1. This method assumes a linear relation between resistance level and gene dosage, which was demonstrated by Uhlin and Nordstrom for ampicillin resistance of the plasmid R1 *drd*19 (30). They found, however, that the resistance to chloramphenicol reached a peak, regardless of further increase in copy number. For P1 Ap Cm and its derivatives, we found the copy number determined from chloramphenicol resistance to be close to that determined by DNA hybridization. The relative ampicillin resistance of P1 copy mutants, on the other hand, was much higher than expected. Although there is no obvious explanation for this, it should be noted that the β-lactamase in P1 is coded by a different transposon (Tn*902*) from that in R1 *drd*-19 and may show a different gene dosage dependence.

The least satisfactory measure of copy number proved to be specific chloramphenicol acetyltransferase and β-lactamase activity. The lack of reproducibility of the data may result from the variable stability of these enzymes in crude extracts.

All of the copy mutants mapped, N3, N20, N22, N24, N26, and E38, are located near each other in the region of the P1 genome containing the plasmid maintenance genes *c*7 (25), *inc*A and *inc*B (28), and *seg* (10, 27), as well as an origin of P1 plasmid replication (M. Stephens and D. Vapnek, manuscript in preparation; M. A. Capage and J. R. Scott, manuscript in preparation). Therefore, the identified genes and sites in P1 that are required for plasmid replication and maintenance are physically clustered, as are those of most other plasmids.

Among the two best-studied plasmids in the Y incompability group, the prophages P1 and P7, two classes of incompatibility behavior have been described (4a). Between two P1s or two P7s, incompatibility is so extreme that no colonies containing both plasmids can be isolated. This severe incompatibility is also observed between P1 or P7 and another group Y member, pIP231 (M. A. Capage, J. K. Goodspeed, and J. R. Scott, in press). Between P1 and P7, however, incompatibilty is less severe (4a). When N100(P7 Cm) is infected with P1 Ap, 5×10^{-2} of the survivors grow on medium containing both drugs. For other plasmids, copy mutants frequently show altered incompatibility. Although the dominance test experiments presented here were not designed to study incompatibility and necessary controls of the frequency of lysogeny for each lysate were not included, approximately the same fraction of double-resistant survivors was obtained when N100(P7 Cm) was infected with a P1 Ap *cop* mutant as with P1 Ap *cop*$^+$. Preliminary experiments involving P1 *cop* infection of a P1 lysogen, however, suggest that at least some of the mutants show greatly reduced incompatibility. Further work should help to elucidate the effect of these mutants on incompatibility.

To examine the regulation of plasmid replication, we tested the *cop* mutants for dominance to wild type. Since colonies containing two P1 plasmids cannot be isolated, a P7 *cop*$^+$ plasmid was used to complement the P1 *cop* mutants. If the P1 *cop* mutation were in a gene which differs in P1 and P7, it would always appear *cis* specific in the complementation test.

We have found that our *cop* mutants fall into two classes; some are *cis* specific, and some are recessive. The first class is easily explained in any of several ways, including that just mentioned. As another explanation, it has been suggested (4a) that there is a site on the host cell required for replication of all plasmids in incompatibility group Y. If this is correct, a plasmid mutant with a higher copy number might result from an alteration in the plasmid site that binds to the host. This type of mutant would also be *cis* specific. In a negative regulatory model for replication (18, 20), a mutation in the plasmid site to which the replication inhibitor binds would also result in *cis* specificity. It is possible that different *cis*-specific *cop* mutants are altered at sites with different functions. Further experiments to determine whether these mutants affect the same function are necessary.

The existence of a class of P1 *cop* mutants recessive to P7 *cop*$^+$ suggests that P1 plasmid replication is under negative control by an inhibitor that is active against both P1 and P7. Such mutants would have defects in either the level or function of the inhibitor. In the temperature-sensitive mutants, presumably either the repressor itself is thermosensitive or synthesis of the repressor is thermosensitive. (A temperature-sensitive P1 *cop* has also been isolated by Stern-

berg et al. [29], but it is not known whether this is recessive to cop^+.) The fact that our temperature-sensitive *cop* mutants are recessive to cop^+ supports the idea of negative regulation for P1 replication.

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