JOURNAL OF BACTERIOLOGY, Feb. 1975, p. 594-599

Vol. 121, No. 2
Printed in U.S.A.

Genetic and Phenotypic Characterization of *dnaC* Mutations

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Received for publication 3 September 1974

The *dna-1*, *dna-2*, *dna-7*, and *dna-28* mutations, all of which are located near min 89.5 on the *E. coli* linkage map, have been characterized further. As previously demonstrated for *dna-2* and *dna-28*, neither the *dna-1* nor *dna-7* mutation affects the ability of a strain to produce bacteriophage λ at temperatures nonpermissive for the continued replication of the bacterial chromosome. The reported temperature-sensitive inhibition of λ production in a strain carrying *dna-7* is shown to be a consequence of a thermosensitive host specificity mutation in the *hsm* gene and not of the *dna-7* mutation. The four *dna* mutations are recessive to the wild type and define a single *dnaC* cistron according to standard complementation criteria. Unlike other characterized *dnaC* mutants, however, strains carrying the *dnaC1* or *dnaC7* alleles exhibit an abrupt cessation of deoxyribonucleic acid synthesis at 42 C that appears to be more compatible with a defect in deoxyribonucleic acid chain elongation rather than in initiation. The possibility that the apparent elongation defect is actually a composite effect of residual synthesis and deoxyribonucleic acid degradation is raised by the net deoxyribonucleic acid degradation observed in the *dnaC1* strain at 42 C. Several alternative possibilities for the function of the *dnaC* gene product are suggested.

Mutations in the *dnaC* region have been assumed to result in defective initiation of rounds of deoxyribonucleic acid (DNA) replication, originally because of results with strain PC2 (4) and, later, an elegant study of strain CT28-3b (13). Several reports of additional mutations mapping in the *dnaC* region have consistently resulted in the conclusion that the mutations are initiation defective (2, 15, 20), and there is evidence for restricted integrative suppression of some *dnaC* strains (2)—a phenomenon that seems logically reserved for initiation mutants (6, 12).

With the exception of *dna-7*, all *dna* mutations in this region, that have been tested, allow λ bacteriophage replication at the nonpermissive temperature (4, 13, 20). Since the ability to support λ growth under conditions nonpermissive for cellular DNA replication was also believed to be a characteristic of initiation-defective, and not elongation-defective, mutants, the *dna-7* mutation was assigned to a separate locus, *dnaD* (17). This decision to designate *dna-7* as the sole representative of the *dnaD* locus was supported further by kinetic evidence suggesting that its defect might be in chain elongation (4).

An earlier report from this laboratory, however, presented strong genetic evidence, though not proof, that the *dna-7* mutation was actually a *dnaC* allele (16). Biochemical evidence that the *dnaC* and *dnaD* in vitro complementing activities could not be differentiated were in agreement with this single-cistron conclusion (18).

This investigation was undertaken to resolve the apparent contradictions raised by these results. The *dna-2* allele defines the *dnaC* locus for the purposes of this analysis.

MATERIALS AND METHODS

Bacteria and bacteriophage. Bacterial strains are listed in Table 1. P1*vir*[s] and λ*c160* were obtained from J. L. Rosner and I. Herskowitz, respectively. λ*c160* was used throughout and is denoted as λ for convenience.

Media. Oxoid nutrient broth no. 2 (Flow Laboratories, Inc.), M9 minimal medium, and P1 medium were used as described previously (17). Lambda broth contained 10 g of tryptone (Difco) per liter and 2.5 g of NaCl per liter; maltose (0.2%) and thymine (20 μg/ml) were added when necessary. Lambda soft agar and bottom agar contained agar at 6.5 g and 11 g/liter, respectively. Labeling medium was M9-glucose supplemented with 20 μg of all amino acids per ml, except methionine (4 μg/ml), thymine (20 μg/ml), thiamine (0.35 μg/ml), deoxyadenosine (200 μg/ml) [^{3}H]thymine (30 μCi/ml), and [^{14}C]methionine (0.25 μCi/ml). [^{14}C]methionine was omitted in single-label experiments.

Genetic techniques. Procedures for transduction and conjugation experiments were as described previ-

TABLE 1. *Bacterial Strains*[a]

Strain	Genotype	Source or reference
PC1	*leu thy dra str dna-1*	4, 17
PC2	*leu thy dra str dnaC2*	4, 17
PC7	*leu thy dra str dna-7*	4, 17
NY56	*leu str dra dnaC2 recA56*	JW1022 × PC2
NY60	*leu str dra dna-1 recA56*	JW1022 × PC1
NY61	*leu str dra dna-7 recA56*	JW1022 × PC7
NY80	*leu thyA str dnaC2*	Spontaneous *dra*$^+$ from PC2
NY81	*leu thyA str dna-7*	Spontaneous *dra*$^+$ from PC7
NY90	*lac rha malB str polA1 tpp*	This paper
NY98	*lac rha malB str polA1 dna-7*	Transduction of NY90
NY105	*leu argG met lac gal str recA56*	16
NY153	*leu str hsm*	Two transductions of PC7
NY164	*lac rha malB str polA1 hsm*	Transduction of NY90
NY169	*lac rha malB str metE hsm*	Transduction of NY164
NY170	*lac rha malB str metE dna-7*	Transduction of NY98
NY171	*lac rha malB str metE dna-7 thy*[b]	Trimethoprim selection of NY170
NY177	*lac rha malB str metE dna-1 thy*[b]	Parallel sequence to NY171
NY178	*lac rha malB str metE thy*[b]	Spontaneous revertant of NY171
NY179	F'501/NY105: *dnaC2 leu*$^+$*/leu argG met lac gal str recA56*	JW1043 × NY105
NY180	F'502/NY105: *dnaC*$^+$ *serB leu*$^+$*/leu argG met lac gal str recA56*	JW1040 × NY105
CT28-3b	*thr leu thi his pro arg thy*[b] *str dna-28*	13
JW227	*thr leu thi his pro arg str recA56 dna-28*	JW1022 × CT28-3b
JW1022	*Hfr KL16 thr ilv recA56 spc*	J. Gross
JW1040	*HfrH serB*	J. S. Parkinson
JW1043	*HfrH dnaC2*	Transduction of JW1040
WI485	*supE*	W. Brammar
HF4704	*E. coli* C prototroph	C. Lark

[a] All strains are *E. coli* K-12 and F$^-$ unless otherwise specified.

[b] All Thy$^-$ strains, except NY80 and NY81, are low-thymine requirers. If it is not known whether a *dra* or *drm* mutation is responsible for the low requirement, no genotype notation is listed.

ously (17). F' episomes were isolated by the method of Low (9) with minor modifications. Trimethoprim selection of Thy$^-$ mutants was by the method of Stacey and Simson (14). Spontaneous thymidine phosphorylase-negative (tpp) mutants were selected as resistant to a mixture of fluorouracil and deoxyadenosine and, subsequently, for their inability to utilize thymidine as a carbon source (1).

Bacteriophage λ infection. To determine the phage yield per input phage during infection of various strains, the methodology was essentially identical to that of Georgopoulos and Herskowitz (5). Bacteria were grown in lambda broth plus maltose to 2×10^8 cells/ml and concentrated twofold in 0.01 M $MgSO_4$. Phage at a multiplicity of approximately 0.1 were adsorbed to the cells for 20 min at room temperature. After adsorption, a sample was removed to determine the number of unadsorbed phage, and two other fractions were diluted 100-fold into Oxoid nutrient broth at 30 and 40 C. These cultures were grown for 105 min at 40 C or 130 min at 30 C, chloroform was added with continued shaking for 10 min, and the mixture was diluted and plated on indicator bacteria. All dilutions were made in suspension medium (18). Indicator bacteria were prepared by centrifuging a culture grown to 5×10^8 to 6×10^8 cells/ml in lambda broth containing maltose and then suspending it in 0.01 M $MgSO_4$.

Efficiency of plating λ phage. Bacteria were grown at 30 or 40 C in lambda broth containing maltose to approximately 5×10^8 cells/ml, centrifuged rapidly, and suspended in 0.01 M $MgSO_4$. Either λ·C or λ·K was added and preadsorbed as above except that adsorption to cells grown at 40 C was done in soft agar. After adsorption the cell-phage mixture was overlaid in soft agar on lambda plates and incubated at the appropriate temperature. λ·C and λ·K denote λ*c160* grown on wild-type *Escherichia coli* C and *E. coli* K-12, respectively.

Complementation experiments. The Rec$^-$ merodiploid strains, grown overnight at 30 C (for CT28-3b, 25 C) in minimal medium, were subcultured into minimal medium and grown into log phase. The cultures were then diluted and plated in duplicate at 30 C (or 25 C) and at 40 C on prewarmed minimal medium.

Incorporation kinetics. Fully labeled cells were prepared by growing cultures at 30 C for 6 to 10 generations in labeling medium to a concentration of approximately 2×10^8 cells/ml. The cultures were diluted into 2 ml of the same medium to give 4×10^7 cells/ml and grown for an additional 90 min at 30 C

with sampling every 15 min to insure that incorporation of both [^{14}C]leucine and [^{3}H]thymine was logarithmic. After 90 min the cultures were shifted to 42 C and samples on Whatman 3 MM filter disks were monitored for trichloroacetic acid-precipitable counts as described previously (17). The dried filter paper disks were counted in a Packard Tri-Carb scintillation counter by using a toluene solution of Omnifluor (New England Nuclear).

Chemicals. Trimethoprim was a gift from S. W. Singleton, Burroughs Wellcome and Co. [*methyl*-^{3}H]thymine and L-[1-^{14}C]methionine were obtained from Schwarz/Mann and Amersham/Searle Corp., respectively. All other chemicals were reagent grade.

RESULTS

Ability of mutants to support λ replication. Strain PC7 was originally reported to be temperature sensitive for DNA replication and for growth of λ bacteriophage (4). During characterization of strain NY98, which had acquired the temperature sensitivity from a *dna-7* strain by transduction, it was noted that λ growth was normal at both the permissive and nonpermissive temperatures. Further tests showed that strain PC7 does indeed produce λ, but that the phage produced plate on the unrestricting *E. coli* C but not on *E. coli* K-12.

The presumption that PC7 carries two temperature-sensitive mutations, one in a *dna* locus and one in the host specificity loci, was verified by their separation. Strain NY98 was derived from PC7 by transduction and, although temperature sensitive for growth, shows characteristic *E. coli* K host specificity at high and low temperatures. Strain NY153, on the other hand, is a temperature-resistant derivative of PC7 (Table 1) and exhibits the altered host specificity of the original mutant.

To define the host specificity defect more clearly, strains exhibiting the temperature-sensitive growth phenotype or the temperature-sensitive host specificity phenotype, and otherwise genetically identical, were analyzed for host-controlled restriction and modification (Tables 2 and 3). The host specificity phenotype of strain NY169, which is temperature resistant for growth, is shown by these data to be $r_K^{\pm}m_K^{+}$ at 30 C and $r_K^{-}m_K^{\pm}$ at 40 C. In agreement with the analysis of Hubacek and Glover (8) and by comparison with strains of similar phenotype analyzed by them, the host specificity mutation is interpreted as resulting in a defective *hsm*-directed protein and is assigned to the *hsm* gene. Strain NY170, which is temperature sensitive for growth, clearly displays a normal m_K^{+} phenotype at both temperatures and is presumably r_K^{+} also, although this can only reasonably be shown at 30 C.

TABLE 3. *Restriction phenotype of mutants*

Strain	Efficiency of plating[a]				Restriction phenotype[b]	
	λC160·K		λC160·C		30 C	40 C
	30 C	40 C	30 C	40 C		
NY169	0.8	0.9	0.007	1.2	$r_K^{\pm}$	r_K^{-}
NY170	1.1		0.0002		r_K^{+}	

[a] Ratio of plaques obtained compared to those obtained on wild-type *E. coli* K-12 (for λ·K) and on *E. coli* C (for λ·C).

[b] $r_K^{\pm}$ denotes a phenotype intermediate to r_K^{+} and r_K^{-}.

Strain NY153 (*dna*$^{+}$, *hsm* in the original mutagenized background) exhibits significantly less restriction at low temperature than does NY169 (*dna*$^{+}$, *hsm* transferred to a different genetic background) (data not shown), and although this difference is not enough to change the phenotype it implies that PC7 may have yet a further alteration in the host specificity genes.

Table 4 shows that PC1 (*dna-1*), PC2 (*dnaC2*), and NY170 (*dna-7*) give good bursts of λ at 40 C, and, thus the *dna-1* and *dna-7* mutations can be added to the mutations in this region that allow vegetative λ growth at the nonpermissive temperature. In addition, only PC7 and some of its derivatives are deficient in host-controlled modification.

Complementation experiments. In an earlier report, data were presented that strongly implied that the *dna-1*, *dnaC2*, and *dna-7*

TABLE 2. *Modification phenotype of mutants*

Strain	Phage production[a] observed on indicator strain				Fraction K-plating phage produced		Modification phenotype[b]	
	E. coli K		*E. coli* C					
	30 C	40 C	30 C	40 C	30 C	40 C	30 C	40 C
NY169	54	0.6	62	177	0.9	0.003	m_K^{+}	$m_K^{\pm}$
NY170	56	198	55	201	1.0	1.0	m_K^{+}	m_K^{+}

[a] Phage yield per input phage.

[b] $m_K^{\pm}$ denotes a phenotype intermediate to m_K^{+} and m_K^{-}.

TABLE 4. *Growth of λ in dna mutants at 40 C*

Strain	Phage[a]
PC1	144
PC2	115
NY170	198

[a] Phage yield per input phage (*E. coli* K-12 as indicator).

alleles were in a single cistron (16). At that time, difficulties encountered in attempts to prove that an episome carried a revertible *dnaC* allele rather than a *dnaC* deletion prevented us from obtaining unequivocal proof that the mutations were all in the same cistron (16).

To circumvent problems of the type previously encountered, a new F′ carrying the *dnaC* region was isolated from an Hfr Hayes strain that contained the *dnaC2* allele. Evidence that this Hfr strain, JW1043, actually carries the *dnaC2* allele is as follows. (i) Temperature sensitivity was introduced by co-transduction with *serB*$^+$ from strain NY80 (*dnaC2*) (Table 1). (ii) JW1043 reverts to temperature resistance with a frequency similar to the observed with PC2, the original *dnaC2* mutant. (iii) No temperature resistant recombinants were isolated from matings of JW1043 with PC2 (*dnaC2*), but such recombinants were obtained when JW1040 (the wild-type parent of JW1043) was mated with PC2, although the number of Leu$^+$ recombinants at 30 C was equivalent in both matings. (iv) Temperature-resistant revertants of JW1043 donated temperature resistance to PC2 in plate matings.

JW1043 (*dnaC2*) and its parent strain JW1040 (*dnaC*$^+$) were mated with NY105, and the F′*leu* containing strains NY179 (F′*dnaC2 leu*$^+$/*leu*) and NY180 (F′*dnaC*$^+$ *leu*$^+$/*leu*) were isolated (Table 1). These F′ strains were used as donors to construct a series of Rec$^-$ merodiploid strains carrying either the *dnaC2* or *dnaC*$^+$ alleles on the episome and *dna-1*, *dnaC2*, *dna-7*, or *dna-28* on the chromosomes.

The data in Table 5 show that *dnaC*$^+$ is dominant to all four chromosomal alleles and that the F′ carrying *dnaC2* fails to complement any of the mutant alleles. Therefore, according to these criteria, all four matations represent lesions in a single *dnaC* cistron.

Incorporation of [^{3}H]thymine at 42 C. There is good evidence that the *dnaC2* mutation results in an apparently initiation-defective phenotype (4), and our own data (not shown) as well as that of several other laboratories are consistent with that interpretation. A mutant carrying the *dnaC28* allele has clearly been shown to be defective in initiation (13). Results with the *dnaC1* and *dnaC7* alleles in strains PC1 and PC7 were not definitive (4; P. Carl, personal communication), possibly because the maximal permissive temperature for the parent of these mutants was 40 to 41 C and, therefore, the mutants could not be tested above these temperatures. Results with PC7, however, suggested that this strain might be defective in elongation (4). Transfer of these alleles to a different genetic background allowed the use of 42 to 43 C as the nonpermissive temperature.

Mutants temperature sensitive for the initiation of DNA replication are expected to show residual synthesis after transfer to the nonpermissive temperature. The total amount of residual synthesis is dependent upon the number of replication forks, which is, in turn, dependent on the rate of growth. For generation times exceeding 40 min, cells are expected to complete each round of DNA replication prior to initiating a subsequent round (7). A sudden inability to initiate new rounds of replication in cultures of such strains should result in incremental residual synthesis of 39% (10).

Incorporation patterns obtained for DNA and protein synthesis with strains NY171, carrying *dnaC7*, and NY177 (*dnaC1*) after a shift from 30 to 42 C are shown in Fig. 1. In this experiment both strains were fully prelabeled and growing with generation times of 55 min (NY171) and 65 min (NY177) at 30 C. The residual increments observed in Figure 1, 25% for NY171 (*dnaC7*) and none for NY177 (*dnaC1*), are significantly less than the 39% expected for initiation-defective mutants. These data, therefore, imply that both the *dnaC1* and *dnaC7* mutations result in

TABLE 5. *Complementation among dna alleles*

Strain	Relevant markers	Total colonies at 30 C[a]	Colonies: 40 C/30 C[b]
F′501/NY56	*dnaC2/dnaC2*	296	$<7 \times 10^{-5}$
F′502/NY56	*dnaC+/dnaC2*	211	0.94
F′501/NY60	*dnaC2/dna-1*	361	$<7 \times 10^{-5}$
F′502/NY60	*dnaC+/dna-1*	1309[c]	0.57–1.2[c]
F′501/NY61	*dnaC2/dna-7*	151	$<7 \times 10^{-5}$
F′502/NY61	*dnaC+/dna-7*	165	0.93
F′501/JW227	*dnaC2/dna-28*	156	$<7 \times 10^{-5}$
F′502/JW227	*dnaC+/dna-28*	142	0.99

[a] Total number of colonies on duplicate plates at one of the dilutions plated.

[b] The actual plating efficiencies are several orders of magnitude lower than 7×10^{-5} and are similar to the reversion frequencies for these alleles.

[c] This strain is the only one that gave significantly varying plating efficiencies at 40 C. Total colonies at 30 C are from two sets of duplicate plates.

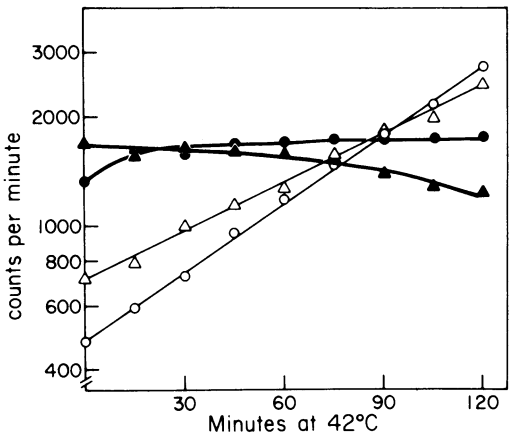

FIG. 1. *Kinetics of DNA and protein synthesis of NY171 and NY177 after a shift from 30 to 42 C. Cultures were prelabeled for approximately 10 generations at 30 C. Strain NY171:* ●, *[^{3}H]thymine;* ○, *[^{14}C]methionine. Strain NY177:* ▲, *[^{3}H]thymine;* △, *[^{14}C]methionine.*

defective DNA chain elongation.

The kinetics of protein synthesis (Fig. 1) demonstrate that the strains are defective specifically in DNA synthesis and also serve as a reference for the rate of incorporation of label into DNA at 30 C. At 30 C, DNA and protein synthesis are parallel and the rate of protein synthesis increases by less than 10% after the shift to 42 C. DNA synthesis in strain NY178 (a spontaneous revertant of the *dnaC7* mutant NY171) shows no perturbation at 42 C (data not shown) and was used as a control in all incorporation experiments.

The apparent breakdown of DNA in NY177 at 42 C was consistently observed in single- and double-label experiments although the extent of breakdown in this strain and the exact amount of residual synthesis in both strains after the shift to 42 C were somewhat variable and apparently influenced by minute differences in water bath temperatures. The DNA synthesis patterns observed at 42 C in strains carrying the *dnaC1* or *dnaC7* mutations are consistent with their being defective in chain elongation rather than in initiation.

DISCUSSION

The complementation results in Table 5 show that the *dna-1*, *dnaC2*, *dna-7*, and *dna-28* mutations are all in the *dnaC* cistron. No intragenic complementation between mutant *dnaC* alleles was observed, and the complete dominance of *dnaC*$^+$—close to 100% as measured by cell viability—implies that the wild-type gene product efficiently replaces the altered *dnaC* products of the mutants. The demonstration that *dna-7* is a *dnaC* allele confirms our earlier evidence (16) and is consistent with the inability to differentiate *dnaC* and *dnaD* complementing activities in vitro (19).

All the *dnaC* mutants that have been tested support the growth of λ bacteriophage at the restrictive temperature (Table 4; and 4, 13, 20). Previous reports of the inability of *dnaC7* to support λ growth have been shown to be a consequence of a temperature-sensitive *hsm* mutation (Tables 2 and 3). In addition, another temperature-sensitive mutation, which confers temperature-sensitive growth and apparently manifests a phenotype only in conjunction with some other mutant gene present in the JW1040 Hfr Hayes background and not in the other genetic backgrounds employed, has been found in PC7 and its derivatives, NY171 and NY178, but not in PC1 or PC2. This mutation is closely linked to *serB* and was discovered during an attempt to transduce *dnaC7* into JW1040. Although this mutation is detected only in the Hfr strain and confers a temperature sensitivity profile noticeably different from that due to *dnaC7*, it is of importance when one is attempting to determine map position. Since NY178 displays normal growth and DNA synthesis at high temperature, this unknown mutation is presumably suppressed in this genetic background.

Finally, although other *dnaC* mutants that have been analyzed appear to be initiation defective (2, 4, 13, 15, 20), the results in Fig. 1 show that two *dnaC* strains synthesize far less than the 39% residual incorporation expected of initiation mutants. These incorporation kinetics for NY171 (*dnaC7*) and NY177 (*dnaC1*) are consistent with their being defective in DNA chain elongation.

Some DNA degradation is observed at 42 C with NY177 (*dnaC1*); as noted previously with *dnaB* mutants, the time of appearance and the extent of degradation vary somewhat from experiment to experiment (17). DNA breakdown in *dnaB* mutants depends, in part, on the action of the *recBC* nuclease, exonuclease V (3), and newly synthesized DNA is preferentially degraded (11).

The residual synthesis observed at 42 C in NY171 (*dnaC7*) varied slightly from day to day, apparently due to minute temperature fluctuations, but generally displayed an abrupt change in rate followed, in some cases, by an extremely low rate of additional incorporation. No DNA degradation was seen in NY171 at 42 C, but it is

possible that DNA breakdown occurred and, by competing with synthesis, resulted in the apparent elongation defective phenotype. DNA breakdown in a *dnaD* mutant has been referred to by Gross (6).

Experiments to characterize the breakdown observed in NY177 (*dnaC1*) and to determine whether breakdown occurs in NY171 (*dnaC7*) are currently in progress. By using [^{3}H]thymidine pulses of NY177 (*dnaC1*), we have, so far, been unable to detect any residual synthesis at 42 C. In spite of this, the apparent defects in chain elongation may not reflect the capacity of the cell to complete rounds of DNA synthesis but rather the vulnerability of the polymerizing DNA to nuclease action. It is, however, not clear how the inactivation of a factor that is involved only in initiation could directly cause the DNA to become vulnerable to a nuclease.

If the *dnaC* product were to function not only as an initiation factor but also throughout the replication cycle, the observed degradation could be explained in several ways. (i) The *dnaC* protein remains at the initiation site and protects an otherwise sensitive DNA configuration from nuclease action; the *dnaC1* or *dnaC7* mutations would then lead to a loss of this protection. (ii) The *dnaC* product is required both for initiation and elongation, and the initiation activity can be inactivated separately (those affecting elongation being the result of more severe lesions). (iii) Although the *dnaC* product functions only in initiation, it is part of a hypothetical replication complex such that its alteration can result in disturbance of the interactions in the complex and indirectly inactivate chain elongation. (iv) The *dnaC* product is a structural component of the hypothetical replication complex, and both the initiation-defective and elongation-defective phenotypes are due to alteration of other activities in the complex.

The last possibility seems unlikely since so many *dnaC* mutants appear to be defective in initiation, and the efficient complementation observed between *dnaC*$^+$ and *dnaC* mutants suggests that, if the *dnaC* product is part of a replication complex, it is not tightly bound in that complex. Experiments are currently in progress to distinguish between these possibilities.

ACKNOWLEDGMENTS

The excellent technical assistance of Shiao-Lien Yang and Leei-Hu Yang is gratefully acknowledged. I am also pleased to thank Ira Herskowitz for introducing me to λ bacteriophage technology.

This work was supported by Public Health Service research grant CA12590 from the National Cancer Institute and by grant GB32417 from the National Science Foundation.

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