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dnaC-Dependent Reconstitution of Replication Forks in *Escherichia coli* Lysates

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Lysates of *Escherichia coli* exhibit a DNA-synthesizing activity that depends on the presence of replication forks and of replication proteins. Replicative activity was reconstituted in vitro by mixing lysates prepared from temperature-sensitive *dnaB* mutants with wild-type *dnaB* protein. Lysates of double mutants deficient in both *dnaB* and *dnaC* genes could only be complemented by the addition of both *dnaB* and *dnaC* proteins, whereas lysates deficient in *dnaC* protein did not require the addition of any exogenous factor. This shows that the replication machinery, once it is running along the chromosome, is independent of *dnaC* protein. *dnaC* activity, however, is required for the replacement of defective *dnaB* protein at running replication forks.

The process of DNA replication in bacteria can be phenotypically divided into two stages. The initiation of a new round of replication controls the formation of two new replication forks at a specific site of the chromosome. The elongation of the daughter molecules is performed progressively by bidirectional fork movement along the chromosome until two complete daughter molecules are synthesized.

Fork movement depends on a series of factors including DNA-binding protein (3), mobile promoter, primase, DNA polymerase III, and elongation factors (4) (for reviews, see references 5 and 21). These proteins, when inactivated, give rise to a quick-stop phenotype in randomly growing cell populations. Inactivation of one of the initiation factors (e.g., RNA polymerase or *dnaA* gene product), however, results in gradually decreasing DNA synthesis (8).

The *dnaC* gene product is difficult to fit into this somewhat simplified scheme. *dnaC* mutants expressing quick-stop as well as slow-stop phenotypes have been described (14, 18). Analysis of the sequence of steps involved in this complex process revealed a role for *dnaC* protein either late in the process of initiation (6, 10, 23) or early in prepriming of discontinuous DNA synthesis (7).

In the present study, we investigated the role of *dnaC* protein in the replication of the bacterial chromosome. We made use of the cellophane disk system, which reflects the activities of a moving replication fork in the absence of initiation of new rounds of replication (12, 13). Essential replication factors can be supplied to the system (11, 20). We prepared the system from *dnaB* mutants, from *dnaC* mutants, and from a *dnaB dnaC* double mutant. A defect in *dnaB* protein led to a system exhibiting strongly reduced replicative activity, which was rescued by complementation with temperature-resistant *dnaB* protein. A single defect in the *dnaC* protein led to no detectable defect in in vitro fork movement. This was expected if *dnaC* protein is considered an initiation factor which is not involved in the continuation of rounds of replication, and if *dnaB* protein is considered an exchangeable fork movement factor.

If, however, lysates of the *dnaB dnaC* double mutant were assayed, we found that effective complementation required *dnaB* protein and, in addition, *dnaC* protein. These results indicate that *dnaC* protein must be present for the addition of thermoresistant *dnaB* protein to lead to reconstitution of the replication machinery. Thus *dnaC* protein belongs neither to the class of factors essential for fork movement nor to the class of initiation factors. Rather, its function is to control formation and reassembly of the replication complex.

MATERIALS AND METHODS

Bacterial strains. All strains used were *Escherichia coli* and are listed in Table 1. All strains except WM485 are K-12 derivatives. All strains except NY177, WM485, and BC1304 require low concentrations of thymine (2 μg/ml).

Preparation of crude protein extracts. Cells were grown at 30°C in PB (17.5 g of Penassay broth [Difco Laboratories] and 2 or 100 mg of thymine per liter) and harvested by centrifugation at 30°C. The following steps were carried out at 0 to 4°C. Wet cell paste (2 g) was washed with 10% sucrose–0.1 M KCl–40 mM Tris-hydrochloride (pH 7.8) and suspended in 2 ml of the same buffer; 0.15 ml of a mixture containing 5 mg of egg white lysozyme per ml, 5% Brij 58, and 40 mM Tris-hydrochloride (pH 7.8) was added. The sample

TABLE 1. Bacterial strains

Strain	Genotype	Comments or reference
CC13	*arg endA polA1 thy*	*arg*, *thy*, F^- derivative of H560 (20)
BT1201	*arg endA polA1 thy dnaB1201*	(20)
BT1304	*arg endA polA1 thy dnaB1304*	(20)
NY177	*lac malB metE rha rpsL thy dnaC1*	(18)
PC1	*leu rpsL thy dnaC1*	(2, 19)
PC2	*leu rpsL thy dnaC2*	(2, 19)
WM485	*B/r arg leu his met pro thy dnaC201*	(14)
BC1304	*arg endA polA1 thy dnaB1304 dnaC201*	(14)
SG1719	*argG gal his1 lac leu malA metB mtl rpsL thy ton xyl dnaC665*	(15)

was gently mixed and quickly frozen at −70°C. After thawing and incubation at 0°C for 1 h the lysate was spun at 160,000 × *g* for 75 min. The supernatant was removed from the spongy sediment and stored at −70°C in working samples. Protein content was ca. 15 mg/ml.

Separation of *dnaB* and *dnaC* activities. Crude protein extract from 20 g of wet cell paste (strain CC13) was prepared as described above, but Brij 58 was omitted (fraction I, 28 ml, 900 mg of protein). Nucleic acids were precipitated with streptomycin sulfate (4%) and removed by centrifugation (15 min, 36,000 × *g*). Protein was precipitated by adding 0.4 g of solid $(NH_4)_2SO_4$ per ml, collected by centrifugation (15 min, 36,000 × *g*), redissolved, and dialyzed against buffer D (2 mM $MgCl_2$, 0.1 mM EDTA, 2 mM 2-mercaptoethanol, 12% glycerol, 20 mM Tris-hydrochloride [pH 7.8]) until the conductivity corresponded to 50 mM NaCl in buffer D (fraction II, 14 ml, 700 mg of protein). A DEAE-cellulose column (Whatman Ltd.; DE 52, 1.6 by 20 cm) equilibrated with 50 mM NaCl in buffer D was loaded with fraction II, washed with 50 mM NaCl in buffer D, and developed with a salt gradient (500 ml, 50 to 1500 mM NaCl in buffer D). *dnaC* activity eluted with the nonbound fraction (20 ml). *dnaB* activity eluted between 300 and 400 mM NaCl. *dnaB* and *dnaC* activities were assayed by complementation of crude mutant lysates analogous to experiments shown in Tables 3 and 4, respectively. Active fractions were pooled, concentrated by dialysis against 60% glycerol and 50 mM NaCl in buffer D, and stored at −18°C. The *dnaC* preparation (fraction III, 6.5 ml, 52 mg of protein) was stable for at least 1 year.

Media, buffers, agar plates, and reaction mixes were as described previously (12).

Pulse-label incorporation (2 min, 0.5 μCi of [^{3}H]thymidine in 0.2-ml samples) was measured in cultures growing in minimal medium complemented with the required amino acids (20 μg/ml) and thymine (2 or 100 μg/ml) as described previously (12).

DNA synthesis in the cellophane disk system has been described (12, 13). Standard conditions for the complementation experiments (Tables 2 through 4) were as follows: 10 min of preincubation on nonradioactive incorporation mix followed by 10 min of incubation on tritiated mix, both at 34°C.

*Eco*RI cleavage pattern analysis of DNA synthesized in vitro was performed as described previously (12).

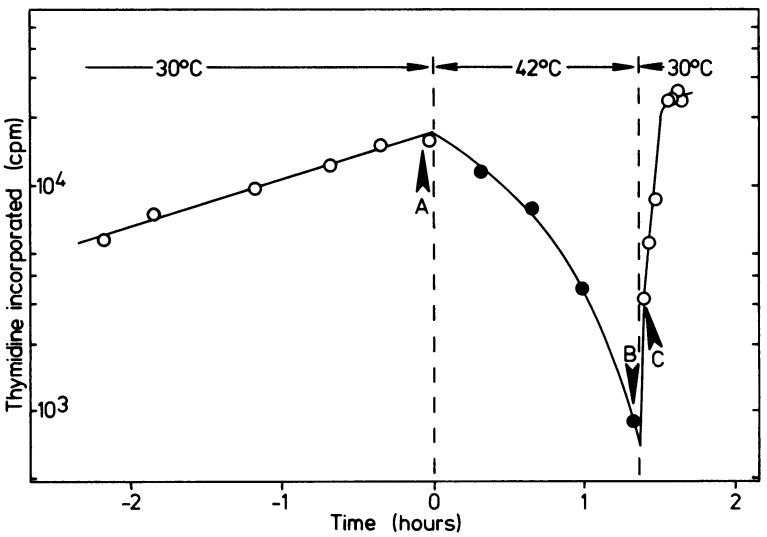

FIG. 1. DNA-synthesizing activity measured as thymidine pulse incorporation into a culture of PC2 (*dnaC2*) at permissive and nonpermissive temperatures and recovery after shift back to permissive conditions. The three arrows indicate the times at which samples have been withdrawn for the in vitro DNA replication analysis shown in Fig. 2.

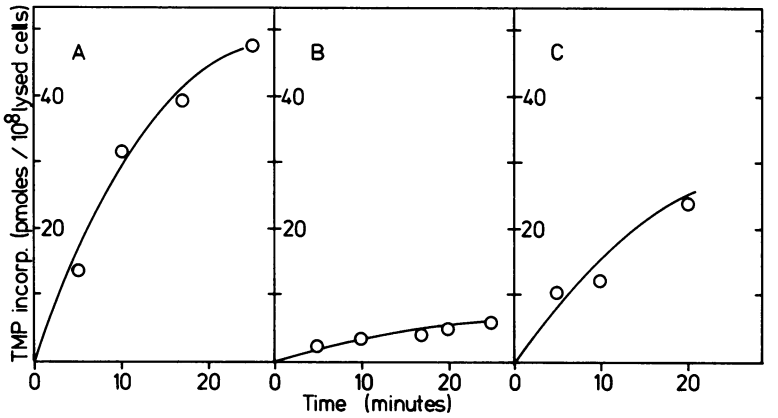

FIG. 2. Kinetics of DNA synthesis at 34°C in lysates of the *dnaC2* mutant PC2. Lysates had been prepared from a culture growing at permissive temperature (A), after cultivation at nonpermissive temperature for 80 min (B), and 2 min after shift back to permissive temperature (C). The corresponding values of the DNA-synthesizing activity in vivo are indicated by arrows in Fig. 1.

RESULTS

Initiation defect of *dnaC* mutants and its effect on DNA synthesis in vitro. The temperature-sensitive strain PC2 (*dnaC2*) exhibits a phenotype typical of initiation defective mutants. DNA synthesis ceases slowly, and a rapid restoration of the DNA-synthesizing capacity is observed after a shift back to the permissive temperature. Figure 1 shows the rate of DNA synthesis during an experimental cycle of growth at the permissive temperature, inhibition after shift to high temperature, and reactivation after shift back to the permissive temperature.

For in vitro analysis, we selected three time points during the course of the experiment. Samples were withdrawn during permissive growth (30°C), at the end of the period at nonpermissive temperature (42°C), and shortly (2 min) after the shift back to 30°C. Cells were lysed on cellophane disks (13), and the DNA replicating activities were determined (Fig. 2). After a long period of inactivation in vivo, we still found some activity in the in vitro system (Fig. 2B). Figure 3 shows the *Eco*RI restriction endonuclease pattern of this residually synthesized DNA. The pattern includes the main fragments obtained from the vicinity of *oriC* (9, 12), which are shown in lane B for comparison. The complexity of the pattern increases with increasing incubation time, e.g., fragments larger than 14 kilobase pairs (kbp) and the 1.87-kbp fragment (12) are not synthesized within the first 5 min. Late fragments such as those between 18 and 23 kbp, one at 10.9 kbp, three between 9 and 10 kbp, and one slightly smaller than 2.24 kbp, were synthesized between the min 15 and 45. The kinetics shown in Fig. 2B excludes the possibility of efficient reinitiation in vitro. Thus some forks, which seem to be released from the initiation block in vivo (during the time the culture was cooled down to 0°C), start in vitro synthesis near *oriC* and move progressively along the chromosome.

Low activity of the *dnaC* mutant in vitro was observed when the culture had been inactivated for a long time before preparation of the system. Absence of inactivation (Table 2), short inactivation (Table 2), and short reactivation (Fig. 2C) result in activities comparable to those found in wildtype lysates. From these data we conclude that the in vitro system resembles the initiation defect of the two strains PC2 (*dnaC2*) and WM485 (*dnaC201*). Fork movement is unimpaired by the temperature sensitivity of the *dnaC* protein. Fork number and fork position depend on the history of the culture used for the preparation of the system.

Complementation of *dnaB* protein depends on *dnaC* protein activity. The in vitro system, when prepared from exponentially growing cells, resembles the activities correlated with fork movement rather than initiation. The use of the initiation mutations *dnaA*, *dnaB252* (23) and *dnaC* (see above) or inhibition of RNA polymerase by rifampin has no inhibitory effect (data not shown). The system depends instead on functioning fork movement factors such as DNA polymerase III, primase, and *dnaB* protein (11, 13, 20). If lysates are prepared from *dnaE* or *dnaG* mutants, activity is low, but is recovered upon addition of the corresponding wild-type protein (11, 20).

Soluble proteins extracted from wild-type cells (see above) showed *dnaB* complementing activity which was absent from extracts prepared from *dnaB* mutants (Table 3). *dnaB* complementing activity was also found in crude

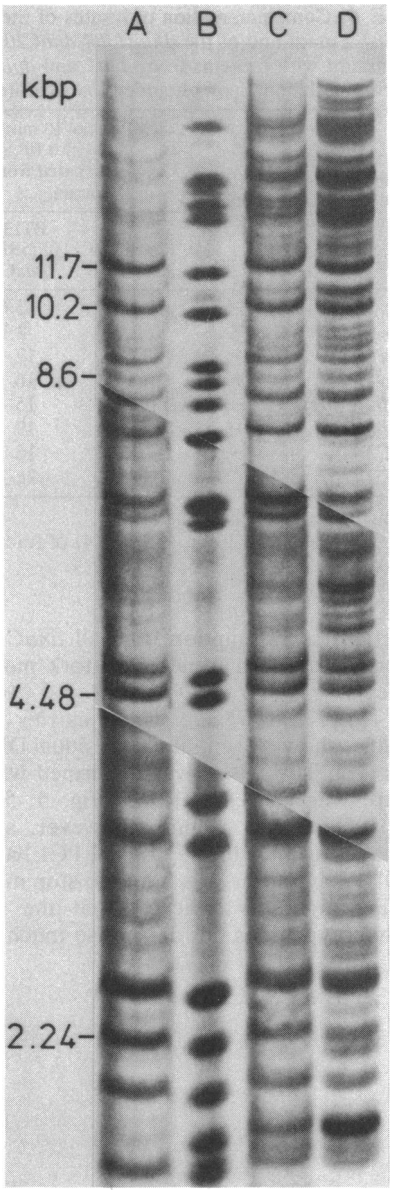

FIG. 3. Size analysis of *Eco*RI digests of DNA synthesized in vitro in lysates of the *dnaC2* mutant PC2. The culture had been inactivated before harvest at 42°C for 80 min. Initiation was allowed for a few seconds during the time of cooling down to 0°C. The amount of DNA synthesized by the preparation is shown in Fig. 2B. In vitro synthesis was for 5 min (lane A), 15 min (lane C), and 45 min (lane D). Lane B shows the *Eco*RI pattern of the vicinity of *oriC* labeled in a culture of *E. coli* synchronized by amino acid and thymine starvation as described previously (12).

proteins extracted from *dnaC* mutants (Table 4). However, when the recipient lysate was prepared from the *dnaB dnaC* double mutant and the complementing preparation was extracted from a *dnaC* mutant, little activity was found (Table 4), although the *dnaC* mutants used were of the initiation-defective type (see above). Complementation of the double mutant was observed only when both temperature-resistant *dnaB* and *dnaC* proteins are present. *dnaC* protein may be provided in the *dnaB* lysate itself, or it may be added in the form of wild-type complementing extract or in the form of partially purified protein (Table 4). The requirement of the double-mutant lysate for both *dnaB* and *dnaC* protein served as an assay system for the partial purification of *dnaC* protein (Fig. 4). Fraction III was essentially free of complementing *dnaB* activity (Table 4), whereas fractions I (Table 3) and II (data not shown) contained considerable amounts of *dnaB* protein.

The distinction between slow- and quick-stop phenotypes is not correlated with special *dnaC* alleles. *dnaC* protein has been described as an initiation factor. In accordance with this idea we found that the *dnaC* mutations of strains PC2 (*dnaC2*) and WM485 (*dnaC201*) did not interfere with fork movement in vitro (Table 2). However, we found that these two mutations interfered with the insertion of wild-type *dnaB* protein into replication forks extracted from *dnaB* mutants (Table 4). This is an effect on forks randomly distributed along the bacterial chromosome, indicating that *dnaC* mutations of the initiation type are able to interfere with fork movement, as soon as the replication machinery at the fork is to be reassembled.

The question remains whether expression of quick-stop phenotypes by *dnaC* mutants favors a second activity of the *dnaC* protein which is directly involved in fork movement (18), or whether this is compatible with an assembly function. Experiments with the cellophane disk system did not give an unambiguous answer, since the *dnaC* mutants PC1 (*dnaC1*), PC7 (*dnaC7*), and NY177 (*dnaC1*) showed a high activity (data not shown) like PC2 and WM485 (Table 2). We therefore reexamined the kinetic

TABLE 2. In vitro DNA-synthesizing activity of mutants carrying the *dnaC2* and *dnaC201* alleles

Lysate prepared from strain	Time of inactivation at 42°C before lysis (min)	dTMP incorporated (pmol/10^8 lysed cells/10 min)
CC13 (*dna*$^+$)		20
WM485 (*dnaC201*)		17
	10	15
	70	2.4
PC2 (*dnaC2*)		30
	80	2.1

TABLE 3. Complementation of *dnaB* mutant lysates depends on temperature-resistant *dnaB* protein in the complementing extract

Lysate prepared from strain	Complementing protein extract[a] prepared from strain:	dTMP incorporated (pmol/10^8 lysed cells/ 10 min)
BT1304 (*dnaB1304*)	Omitted	3.6
	CC13 (*dna*$^+$)	17
	BT1304 (*dnaB*)	7
	BT1201 (*dnaB*)	8
BT1201 (*dnaB1201*)	Omitted	1.9
	CC13 (*dna*$^+$)	16
	BT1304 (*dnaB*)	2.6
	BT1201 (*dnaB*)	2.7

[a] Two microliters per disk.

TABLE 4. Complementation of lysates of the *dnaB1304* mutant and of the *dnaB1304 dnaC201* double mutant with proteins from *dna*$^+$ and *dnaC* strains and the dependency on added *dnaC* protein

Complementing protein extract[a] prepared from strain:	*dnaC* protein[b] added	dTMP (pmol/10 min) incorporated into 10^8 lysed cells prepared from strain:	
		BC1304 (*dnaB1304 dnaC201*)	BT1304 (*dnaB1304 dnaC*$^+$)
Omitted	−	1.7	3.6
Omitted	+	2.8	3.7
CC13 (*dna*$^+$)	−	11	17
CC13 (*dna*$^+$)	+	10	16
WM485 (*dnaC201*)	−	2.1	15
WM485 (*dnaC201*)	+	11	19
PC2 (*dnaC2*)	−	1.7	16
PC2 (*dnaC2*)	+	14	21

[a] Two microliters per disk.
[b] Saturating amounts (20 μg, see Fig. 4) of fraction III per disk.

behavior of these mutants and found that mutants previously assigned to the quick-stop family, including PC7 (*dnaC7* [18]), SG1719 (*dnaC665* [15]; Fig. 5), and PC1 (*dnaC1*; Fig. 5) were of the slow-stop type. Strain NY171, which carries the *dnaC7* mutation transduced from PC7, shows a 25% increase in DNA content after shift to the nonpermissive temperature (18). In contrast to the argument of Wechsler et al. (18), this figure is consistent with a defect in initiation, if the duration of one round of replication and the doubling time are assumed to be 40 and 60 min, respectively (25% can be calculated by inserting n = 40/60 into equation 19 of reference 17). An increase of 25% in any case is too high for a quick-stop mutant. These data favor the simple assumption, that all *dnaC* alleles express initiation rather than fork movement defects.

Strain NY177, however, which carries the *dnaC1* allele, shows essentially no residual DNA synthesis (18). This result was confirmed when the culture was shifted to 45°C (Fig. 5; 50% occurred in less than 2 min). However, at a lower temperature, or in the original PC1 background DNA, synthesis was found to stop more slowly (Fig. 5). This indicates that the observed phenotype does not depend so much on

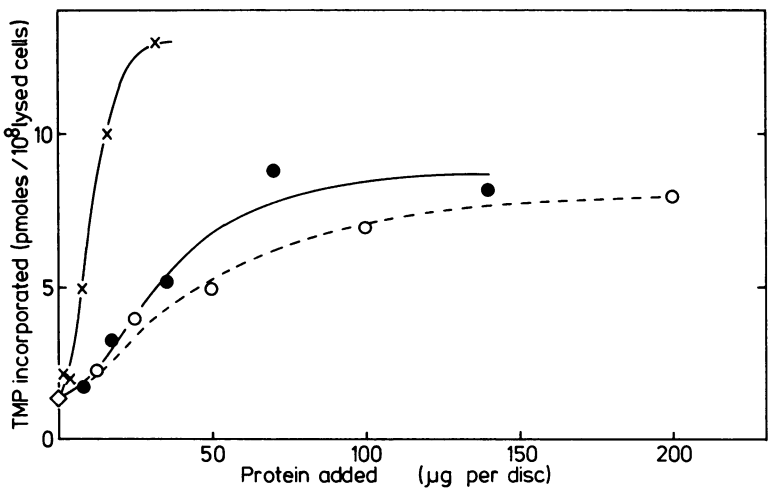

FIG. 4. Dependency of the *dnaB* complementation system on the addition of temperature resistant *dnaC* protein. A lysate (ca. 5 × 10^7 cells) of the double mutant BC1304 (*dnaB dnaC*) was mixed on cellophane disks with crude protein extracts (2 μl) prepared from strain PC2 (*dnaB*$^+$ *dnaC2*) and with various amounts of protein fractions prepared from strain CC13 (*dna*$^+$). Symbols: O, fraction I (crude protein extract); ●, fraction II (ammonium sulfate); ×, fraction III (DEAE-cellulose).

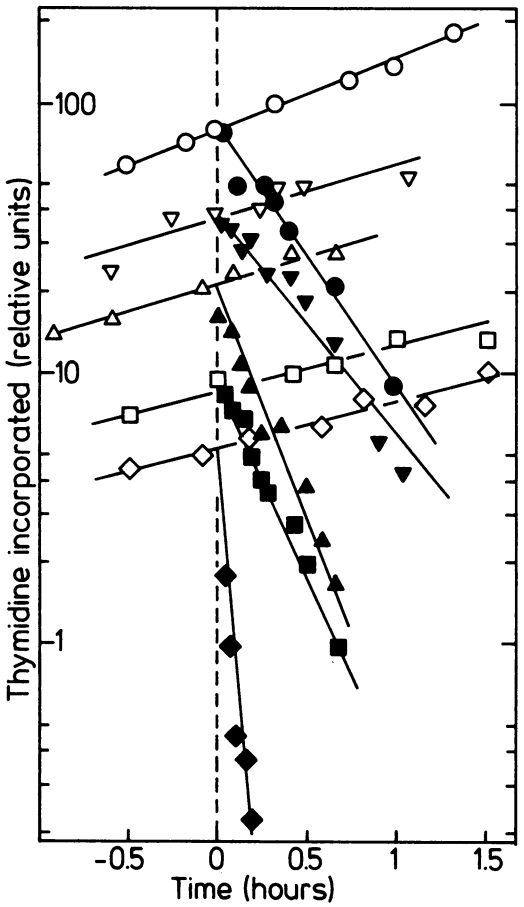

FIG. 5. DNA-synthesizing activity in cultures of *dnaC* mutants at permissive temperature (30°C, open symbols) and after shift of part of the cultures to high temperature (solid symbols). Symbols: (○, ●) SG1719 (*dnaC665*), 42°C; (▽, ▼) PC1 (*dnaC1*), 42°C; (△, ▲) PC1 (*dnaC1*), 45°C; (□, ■) NY177 (*dnaC1*), 42°C; (◇, ◆) NY177 (*dnaC1*), 45°C.

the allele employed, but rather on secondary factors such as genetic background, temperature, and growth conditions.

Although the kinetic data presented are not a strong argument, they are at least consistent with the idea that there is only one class of *dnaC* alleles that in general express initiation-type defects. If fork movement deficiencies are observed, they result from a combination of the *dnaC* defect and some other instability.

DISCUSSION

dnaC protein apparently plays a dual role in DNA replication since *dnaC* mutants exhibit initiation defects as well as fork movement defects. The question is whether these two different phenotypes are due to different functional sites of the protein.

Some evidence has already been presented indicating that the differentiation of *dnaC* alleles into initiation-defective types and fork movement-defective types is not valid: minus strand synthesis of phage φX174 and replication of ColE1 DNA are impaired in protein extracts prepared from either type of *dnaC* mutants (16, 22).

In the present study we used slow-stop *dnaC* mutants and showed that the mutations interfere with in vitro DNA replication in two different ways: *dnaC* activity regulates the number (Fig. 2) and position (Fig. 3) of replication forks, depending on the time course of nonpermissive and permissive temperatures applied during cultivation of the cells before lysis. This indirect effect reflects the initiation-type defect. It does not interfere with fork movement in vitro (Table 2). In addition *dnaC* activity controls the complementation of a defective lysate by temperature-resistant *dnaB* protein (Table 4). This is an effect on fork movement and is independent of the initiation of new rounds of replication. Schuster and co-workers presented similar results obtained in vivo with the *dnaB dnaC* double mutant. Their strain carries a P1 *bac* phage which suppresses the *dnaB* lesion by the activity of *ban* protein. This partially suppressed double mutant expressed the quick-stop phenotype, although the original *dnaC* mutant shows the slow-stop phenotype (14). Combined with kinetic studies on some of the quick-stop mutants (Fig. 5), these results show that the alleles under investigation result in initiation as well as fork movement defects. What is the function of *dnaC* protein in the various DNA replicating systems? The studies by Kornberg (5) and his co-workers on φX174 DNA replication indicate a role for *dnaC* protein in the formation of a prepriming complex. This primosome consists of at least protein n′ and *dnaB* protein bound to the DNA. It moves along the template and is reused in successive cycles of complementary strand synthesis without reconstitution (7).

The situation in the replication fork seems to be quite similar: *dnaC* protein is absolutely required for initiation, i.e., for the formation of the replication complex. This replisome contains bound *dnaB* protein (11). It travels along the DNA without requiring the action of *dnaC* protein. If, however, new *dnaB* protein molecules have to be inserted into the system once it is running, the presence of *dnaC* protein is required again.

It is unclear which selective pressures resulted in the complicated chain of prepriming and priming reactions which include at least the following steps: binding of *dnaB* protein to the

template catalyzed by several factors, including *dnaC* protein; promotion of *dnaG* primase by bound *dnaB* protein; and synthesis of a primer required for the action of DNA polymerase. As pointed out by Alberts and Sternglanz (1), it is for the sake of extreme copying fidelity that chain priming and chain elongation are steps performed by different enzymes. But what is the indispensable function of the prepriming factors? Certainly they are not an absolute requirement for DNA replication since some small phages such as M13 or G4 perform without these factors. The closely related ϕX174 does make use of them, most probably because they are provided by the host cell. The genome of the host cell, however, differs from the small phage genomes in that it is a long molecule requiring an extended replication time. A replisome, once sent on its way at *oriC*, has to remain functionally intact for about 40 min until it reaches termination. If it is damaged on its way, the forked chromosome may become a lethal factor for the cell, unless the replisome is reconstituted. This might be the reason why subunits of the putative replisome are floating in the cytoplasm. Reconstitution requires the presence of recruitable factors. Reassembly of replisomes requires an effective control as well, since uncontrolled formation of replisomes at sites of transcription, repair, or recombination would result in the production of forked chromosomes and might be lethal in the same way that an irreversibly damaged replisome is lethal. It is the circular organization of the chromosome that has to be conserved in any case. Thus reconstitution of a replisome needs to be catalysed by special guiding factors. *dnaC* protein is apparently one of these controlling factors.

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