Mechanism of Replication of ϕ X174 Single-Stranded DNA

IX. Requirement for the *Escherichia coli* *dnaG* Protein

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Escherichia coli NY73, possessing a temperature-sensitive mutation in the *dnaG* locus, was rendered sensitive to bacteriophage ϕ X174 by P1 transduction. ϕ X174 reproduces in this strain at 30 C but not at 40 C. All three stages of ϕ X174 replication, parental replicative form (RF) synthesis, RF replication, and progeny single-stranded DNA synthesis, are thermolabile in this mutant. Competition-annealing data show that both plus- and minus-strand synthesis are equally inhibited after shift up to 40 C during RF replication. We conclude that the *dnaG* gene product is required for the synthesis of both strands of ϕ X RF during RF replication and of the complementary strand and viral progeny strands during stages I and III, respectively.

Mutations causing *Escherichia coli* to be temperature sensitive for DNA replication have been mapped at a number of different loci on the genetic map (for review see reference 14). The products of the *dnaE* and *dnaF* genes have been identified as DNA polymerase III (11) and the B₂ subunit of ribonucleotide reductase (10), respectively, whereas those of the *dnaA*, *dnaB*, *dnaC(D)* and *dnaG* loci have yet to be associated with specific in vivo functions. These *dna^{ts}* mutants encompass three types of defects in DNA synthesis: the initiation of rounds of replication, chain propagation (which includes Okazaki fragment initiation and/or elongation), and precursor synthesis. The first category is characterized by a gradual shut-off of bacterial DNA synthesis, whereas the latter two are of the quick-stop variety. *E. coli* carrying a *dnaG^{ts}* allele possess the second phenotype involving chain propagation and exhibit little or no host synthesis within minutes after shift-up to the nonpermissive temperature (29).

Starting with the premise that studies of the replication of small DNA molecules like that of ϕ X174 will be useful in determining the functions of some of these as yet unidentified proteins, we initiated a study of the in vivo consequence of the *dnaG* lesion on ϕ X DNA replication. Such in vivo studies have already been reported for a number of different *dna^{ts}* hosts (5, 8, 13, 17, 26). For this work the ϕ X174 receptor locus was introduced into an *E. coli* *dnaG* strain, NY73, by P1 transduction. We report in this paper the effect of the *dnaG* mutation on the replication of ϕ X174.

MATERIALS AND METHODS

Selection of the ϕ X⁺ *dnaG* strain. *E. coli* NY73 (*leu⁻*, *metE⁻*, *thy⁻*, *polA1*, *rif^r*, *str^r*, *dnaG^{ts}*) is a *polA1* derivative (3) of PC3, a *dna^{ts}* mutant previously assigned to the *dnaG* locus (29) but which is resistant to all strains of ϕ X174 tested. It was rendered ϕ X⁺ by a two-step transduction with P1kc. An *xyl⁻* mutation (the *xyl* locus at 70 min cotransduces with the ϕ X adsorption locus) was introduced into NY73 from CR34 (*F⁻*, *thr⁻*, *leu⁻*, *thy⁻*, *xyl⁻*) with P1kc. The transduced cells were grown at 30 C for 30 min in minimal medium with xylose, and then penicillin was added to a final concentration of 2,500 U/ml. After 1 h at 30 C, the cells were washed two times in cold 0.05 M citrate (pH 5.5) and allowed to grow up in minimal glucose medium. The xylose selection procedure was then repeated. The surviving cells were then plated on MacConkey plates with xylose as the carbon source. Some of the white colonies growing up on these plates at 30 C were shown to be xylose-requiring in liquid culture medium and to retain their temperature sensitivity for DNA synthesis. The ϕ X174 sensitivity locus was then introduced into this NY73 *xyl⁻* strain from *E. coli* strain CR (ϕ X⁺, *suIII⁺*, *str^r*, *thy⁻*) and NY73 *xyl⁺* colonies isolated on minimal xylose plates. One out of 13 tested *xyl⁺* colonies was sensitive to ϕ X174, did not suppress ϕ X174 amber mutations, and was temperature sensitive. This strain will be referred to as NY73S in this paper. A spontaneous temperature-resistant revertant of this strain was isolated and designated NY73SR.

The kinetics of inhibition of host DNA synthesis of NY73S at the nonpermissive temperature was indistinguishable from the parent NY73. Incorporation of [³H]thymidine into acid-precipitable material was reduced by greater than 90% after 10 min at 40 C or 5 min at 42 C. The presence of the *dnaG* mutation was confirmed by mating NY73S with *E. coli* F⁺102

(*argG*⁺/*argG*⁻, *his*⁻, *leu*⁻, *metB*⁻, *recA1*, *lac*⁻, *mal*⁻, *xyl*⁻, *str*^r) and showing that NY73S *ts*⁺ recombinants were generated at high frequency (29).

Other *E. coli* and ϕ X174 strains as well as standard procedures have been described (6). ϕ Xam3 is lysis defective as a consequence of a mutation in cistron E.

Preparation of ϕ Xam3 labeled with [³H]thymidine at a specific activity of about 10⁻⁶ counts per min per PFU has been described (4). NY73 was obtained from H. Hoffmann-Berling, F'102 (JC1553) from J. Wechsler, and CR34 from D. Lane.

Pulse labeling, cell lysis, and pronase-sodium dodecyl sulfate digestion. Cells (NY73S or NY73SR) were grown to 2 × 10⁸ to 4 × 10⁸ cells/ml in mT3XD (21) with aeration by bubbling.

Cultures were labeled with [*methyl*-³H]thymidine added to 1 μ Ci/ml. The pulses are routinely halted by diluting in an equal volume of a solution containing 75% ethanol, 20 mM sodium acetate, pH 5.5, 4 mM EDTA, and 2% phenol saturated with 0.05 M sodium tetraborate (20). The cells were washed, lysed with lysozyme-EDTA at 0 C, and digested with Pronase and sodium dodecyl sulfate at 37 C, and the bacterial DNA was precipitated out at 4 C in the presence of 1 M NaCl as described by Schekman et al. (21).

Centrifugation. Neutral sucrose gradients (linear 5 to 20% sucrose, 1 M NaCl, 50 mM Tris-hydrochloride, 2 mM EDTA, pH 7.5) were centrifuged in the SW 56 (55,000 rpm for 180 min at 15 C) or in the SW 50.1 (50,000 rpm for 180 min at 15 C) in the Beckman L265B ultracentrifuge. CsCl equilibrium density gradients (1.25 g of CsCl per g of solution, density 1.71 g/ml) were centrifuged for 48 h at 40,000 rpm in the A321 rotor of the IEC B60 ultracentrifuge at 15 C in 10 ml of polyallomer tubes. Samples of the gradients were taken onto 1-inch (about 2.54 cm) squares of Whatman no. 5 paper, dried, and counted in 10 ml of toluene Omnifluor (New England Nuclear Corp.) with the appropriate settings in an Intertechnique SL 36 scintillation spectrometer.

Competition annealing. The annealing of labeled ϕ X174 DNA in the presence of increasing concentrations of unlabeled plus strands has been described (9). [³H]-labeled replicative form (RF) was combined with unlabeled RF to give a final concentration of 8 to 10 μ g/ml, sonicated, denatured by heat, supplemented with different excess concentrations of unlabeled plus-strand DNA extracted from virions, and reannealed to give a C₀t in excess of 1. Samples were digested with the *Neurospora crassa* single-strand specific nuclease, and the amount of renatured duplex DNA determined by precipitation with trichloroacetic acid.

Reagents and buffers. Phage dilutions were made in 0.05 M sodium tetraborate. mT3XD is the standard medium for all the experiments (21). [*Methyl*-³H]thymidine (40 to 60 Ci/mmol) was obtained from New England Nuclear Corp. Penicillin G, the potassium salt, (1,585 U/mg) were from Calbiochem. MacConkey-xylose plates were made from MacConkey base (Difco) supplemented with xylose to a final concentration of 0.2%.

Liquid minimal medium contains M9 minimal salts (1) supplemented with 1 μ g of vitamin B₁ per ml,

50 μ g of leucine per ml, 25 μ g of methionine per ml, 10 μ g of thymine per ml, and either glucose or xylose to 0.2% final concentration. Minimal plates were poured with one-half volume sterilized 4% agar (Difco) and one-half volume of liquid minimal medium containing twice the concentrations of nutrients indicated above.

Other. The procedure for measuring intracellular phage has been described (6). [³²P]RF was made as described by Schekman et al. (21).

Transductions with P1kc essentially followed the method of Lennox (19). Matings with F'102 were performed at 30 C in L broth (19) by the method of Adelberg and Burns (2).

RESULTS

Phage growth in NY73S. The effect of the *dnaG* mutation on the growth of ϕ X174 was tested at 30 and 40 C. Figure 1A shows the effect of a temperature shift-up on the generation of progeny phage. NY73S was infected with ϕ Xam3 at 30 C and after 3 h the growth cycle was complete; at this less than optimal temperature the burst size (intracellular phage yield) was still rather small, under 100 PFU per cell. When the culture was shifted to 40 C 5 min after infection, there was a drastic reduction in

FIG. 1. Growth of ϕ X174am3 in NY73S and NY73SR at 30 and 40 C. Cultures of NY73S and NY73SR were grown to 2 × 10⁸ cells/ml in mT3XD at 30 C. At zero time they were infected with ϕ Xam3 at a multiplicity of 5. One-half of each of the cultures was shifted to 40 C after 5 min. At various times samples were withdrawn and intracellular PFU per milliliter was determined (6). Panel A: NY73S; Panel B: NY73SR. Symbols: (●) 30 C; (○) 40 C.

the burst size. If the shift to the higher temperature was made at the same time as infection or if the shift was to 42 C, the burst size was reduced to under 0.1 PFU per cell (data not shown). Panel B shows that ϕ X174 growth in NY73SR, a spontaneous *dnaG*⁺ revertant of NY73S, was not inhibited by the shift to 40 C. This is evidence that the inhibition of phage synthesis at the high temperature was a direct consequence of the *dnaG* mutation.

The replicative cycle of ϕ X174 involves three resolvable stages; conversion of the input single-stranded circular DNA to a duplex (RF), semi-conservative replication of this parental RF to produce a pool of progeny RF, and finally the asymmetric displacement of the viral (designated plus) strands from these RF molecules to generate single-stranded viral DNA molecules which are rapidly encapsulated into mature virions. The purpose of the subsequent experiments was to determine the temperature sensitivity of these synthetic events in the *dnaG* host.

Parental RF synthesis. One direct way of demonstrating the incorporation of a single-stranded DNA molecule into a duplex structure is by isopycnic centrifugation in neutral CsCl equilibrium density gradients; single- and double-stranded ϕ X174 DNA have buoyant densities of 1.725 and 1.708 g/ml, respectively. In the experiment presented in Fig. 2A, a culture of NY73S at 30 C was infected with [³H] ϕ X; 10 min later the viral DNA was purified and centrifuged to equilibrium in a CsCl density gradient with marker [³²P]RF. Most of the [³H]DNA banded with the marker indicating that efficient conversion of the viral DNA to parental RF had occurred at 30 C. When the period of infection was reduced to 5 min, a large proportion of the ³H appeared at a density intermediate between single- and double-stranded DNA. The presence of this heterogeneously banding intermediate density material, which normally does not appear to any great extent in infections of wild-type cells and is presumably partially formed RF, suggested that parental RF synthesis is somewhat retarded even at 30 C (G. McFadden, unpublished data).

When the host cells were preincubated for as little as 10 min at 40 C before infection, no [³H]RF was made (Fig. 2B), indicating that parental RF synthesis has an obligatory requirement for the *dnaG* product *in vivo*. This observation is consistent with the *in vitro* temperature sensitivity of the reaction in an NY73 lysate (30).

RF replication. Since ϕ X174 did not grow well in NY73S at 30 C, the question of whether

Fig. 2. Neutral CsCl equilibrium centrifugation of ϕ X DNA from NY73S infected with [³H] ϕ X174am3. A 20-ml culture of bacteria was grown at 30 C to a cell density of 4×10^8 cells/ml in mT3XD. Ten minutes before infection, one-half of the culture was shifted to 40 C. [³H] ϕ X174am3 was added to both at a multiplicity of 3. After 10 min, both infections were stopped with an equal volume of ethanol-phenol and the viral DNA was purified as described. The total intracellular viral DNA was further purified by sedimentation velocity centrifugation in a neutral sucrose gradient and then centrifuged to equilibrium in a neutral CsCl gradient with [³²P]RF marker in the IEC A321 rotor. Panel A: 30 C; Panel B: 40 C. Density increases towards the left. Symbols: (●) ³H; (○) ³²P.

RF replication was thermolabile in NY73S was more readily asked in cultures treated with 30 μ g of chloramphenicol per ml to prevent the onset of single-stranded DNA synthesis (25). Thus, a culture of ϕ X-infected NY73S in chloramphenicol was shifted to 40 C and 5 to 10 min later labeled for 1 min with [³H]thymidine. A sample kept at 30 C was similarly labeled. The viral DNA was purified on neutral sucrose gradients; the amount of radioactivity cosedimenting with marker [³²P]RF (after correction for the low level of incorporation in uninfected cells) is given in Table 1. RF synthesis was dramatically reduced within minutes after the shift. In the second experiment in Table 1 the

TABLE 1. RF replication in ϕ X-infected NY73S at 30 and 40 C

Temp and time before pulse	[³ H]RF synthesis ^a	
	With chloramphenicol ^b	Without chloramphenicol ^b
30 C only	95,700 (100%)	6,530 (100%)
5 min at 40 C	1,960 (2.0%)	
10 min at 40 C	340 (0.3%)	130 (1.9%)

^a ϕ Xam3 was added to a multiplicity of 5, and 5 min later chloramphenicol was added to 30 μ g/ml. Ten-milliliter portions were shifted to 40 C either 5 or 10 min before the pulse. In the culture treated with chloramphenicol, the 1-min pulse of [³H]TdR was given at 40 min after infection; in the culture without chloramphenicol the pulse was given at 15 min. Labeling was terminated with ethanol-phenol, and the viral DNA was extracted and sedimented through a neutral sucrose gradient with [³²P]RF marker as described. The numbers shown are the ³H counts per min cosedimenting with the [³²P]RF after correction for contaminating host cell DNA sedimenting in this region.

^b The figures in parentheses represent the percent of synthesis relative to the 30 C value.

same observation was made in the absence of chloramphenicol.

To determine whether there was preferential inhibition of synthesis of either the plus or the minus strand of ϕ X at 40 C, a culture of infected NY73S was shifted to the restrictive temperature for only 2 min before a 1-min [³H]TdR pulse. At this time there is still about 40% of the incorporation into ϕ X RF as at 30 C. The pulse-labeled DNA was purified on a neutral sucrose gradient, banded preparatively in a neutral CsCl equilibrium density gradient, and analyzed by annealing with cold RF (after sonication and denaturation) in the presence and absence of excess unlabeled plus-strand DNA. If either plus- or minus-strand synthesis had been preferentially inactivated during the 2-min shift-up time, then the fraction of pulse label which reannealed in the presence of viral strand competitor should have either increased or decreased, respectively. However, the fraction of label that was incorporated into each strand during the pulse was the same at 30 and 40 C (Table 2). Therefore we infer that the *dnaG* gene product is required for the synthesis of both plus- and minus-strand DNA during RF replication.

Progeny single-stranded DNA synthesis. The final stage of the viral life cycle is the exclusive synthesis of plus strands for encapsulation into progeny virions. This terminal event was also temperature sensitive in NY73S (Fig. 3). A portion of a culture infected with ϕ Xam3

at 30 C was labeled with [³H]thymidine for 1 min during single-stranded DNA synthesis. Portions of the same culture, which had been shifted to 40 C for 5 or 10 min, were similarly labeled with [³H]thymidine. The sedimentation velocity profiles of the ³H-labeled viral DNA in neutral sucrose gradients (Fig. 3) show that the incorporation of [³H]TdR into ϕ X DNA was reduced by more than 90% within 10 min at the high temperature (a 30 C culture continues to synthesize viral material for over 90 min [Fig. 1]). Most of the [³H]TdR incorporated in the 1-min pulse was found in RF II and more rapidly sedimenting intermediates because progeny viral DNA synthesis proceeds via an asymmetric displacement of plus strands from RF (7); only after longer periods of synthesis (under permissive conditions) does label accumulate in viral plus strands. The conclusion is that the *dnaG* product is required for progeny single-stranded ϕ X DNA synthesis.

DISCUSSION

ϕ X replication in vivo has been studied extensively in *E. coli* strains carrying the *dnaB* (26), *dnaC* (17), and *dnaE* (5, 8, 13) lesions. ϕ X-resistant cells treated with Ca²⁺ become susceptible to transfection by ϕ X174 single- or double-stranded DNA (27), and in this system ϕ X growth has been shown to be temperature sensitive in cells with *dnaB*, *dnaC*(D), *dnaE*,

TABLE 2. Competition annealing of [³H]RF pulse-labeled in NY73S during RF replication^a

Pulse temp (C)	Uncompeted ³ H label ^b	Six-fold excess competitor ^b	36-fold excess competitor ^b
30	17,100 (1.0)	9,300 (0.54)	9,150 (0.53)
40	7,450 (1.0)	4,350 (0.58)	4,100 (0.55)

^a ϕ X174am3 was added to a multiplicity of 5. After 5 min chloramphenicol was added to give a concentration of 30 μ g/ml. At 40 min after infection one-half (30 ml) of the culture was shifted to 40 C. Two minutes later, both portions were labeled for 1 min with [³H]TdR. Pulses were stopped with ethanol-phenol, the viral DNA was extracted and purified by neutral sucrose gradient sedimentation and CsCl banding. Competition annealing in the presence of 6- and 36-fold excess plus strand was performed as described. Uniformly labeled [³²P]RF was present as a control. Identically treated uninfected NY73S cultures were treated as above and the values (a small fraction of the total) were subtracted. Determinations were done in duplicate and the average value is presented. In both competitions about 41% of the ³²P present in uniformly labeled [³²P]RF added as a control was competed out by the 36-fold excess plus strands.

^b Figures in parentheses give the fraction of label which was not competed out by the excess plus-strand material.

FIG. 3. Neutral sucrose gradient sedimentation of viral DNA from ϕ X174am3-infected NY73S pulse labeled with [3 H]TdR during progeny single-stranded DNA synthesis. A 30-ml culture of bacteria was grown to 4×10^8 cells/ml in mT3XD at 30 C. ϕ Xam3 was added to a multiplicity of 5. Two 10-ml samples were shifted to 40 C at 5 and 10 min before the pulse. At 55 min after infection the three 10-ml portions were labeled with [3 H]TdR for 1 min, stopped with ethanol-phenol, lysed, digested with Pronase-dodecyl sulfate and centrifuged in a neutral sucrose gradient as described. The arrow indicates the position of a [32 P]RF II DNA marker. Sedimentation is towards the left. Symbols: (●) 30 C; (○) 5-min shift at 40 C; (□) 10-min shift.

and *dnaG* genotypes but not in those with *dnaA* or *dnaF* genotypes (28). In vitro, a cleared lysate which efficiently performs the first synthetic event of ϕ X174 replication, the synthesis of the parental RF molecule, has been described; for parental RF formation in this system there is a requirement for the gene products of the *dnaB*, *dnaC(D)*, *dnaE*, and *dnaG* loci (22, 30). There is some ambiguity over the *dnaA* gene requirement in this system (22, 33) and also a discrepancy in the observations that parental RF synthesis in *dnaC* (17) and *dnaE* (5, 8, 13) hosts is temperature resistant in vivo but not in vitro (30); in contrast, our in vivo results implicating the *dnaG* product in parental RF formation agree with the in vitro results.

Although the function of the *dnaG* protein is not understood, some evidence from in vitro systems has been published suggesting that it is involved in the initiation of Okazaki fragments in *E. coli*. When NY73 was lysed on a cellophane disk and newly synthesized intermediates were analyzed, Lark (18) concluded that fragments initiated at the permissive temperature were elongated at the high temperature but no new initiations occurred. It has also been reported that in the cleared-lysate system previously mentioned the *dnaB*, *dnaC(D)*, and *dnaG*

functions are involved in the RNA priming event but not in the subsequent elongation reaction (32). This is consistent with the observation that the synthesis of *oop* RNA required for λ replication requires the *dnaG* gene (15). Fork propagation during colicinergic factor E1 replication is arrested at 42 C in a *dnaG* host (BT 308); but whether this involves a defect in a chain initiation event or not is unclear (D. Helinski, personal communication). It should also be mentioned that the *dnaG* protein has been purified by the use of complementation assays, but no particular activity has yet been reported for the purified product (16, 31).

Since the rolling circle model of ϕ X174 replication (12), for which there is some in vivo evidence (23, 24), predicts a dissimilarity in the initiation reactions for the plus and minus strands (the first being at a nicked site on the double-stranded RF template, the second being de novo on the displaced single-stranded tail), we might expect at least some protein(s) to be involved with the priming of one strand but not the other. The data presented here indicate that the *dnaG* protein is probably not one of these. Not only is synthesis of both plus-strand DNA during single-strand synthesis and minus-strand DNA during parental RF formation temperature sensitive in NY73S, but [3 H]thymidine incorporation into both strands is inhibited to the same extent during a short shift-up period during RF replication. Should the *dnaG* protein prove to be required for synthesis of an RNA primer for DNA synthesis, then the observations in this paper showing *dnaG* involvement in all three stages of ϕ X174 replication would indicate that this event is a common feature of both plus- and minus-strand synthesis.

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