

Physiological Effects of Growth of an *Escherichia coli* Temperature-Sensitive *dnaZ* Mutant at Nonpermissive Temperatures

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The physiological effects of incubation at nonpermissive temperatures of *Escherichia coli* mutants that carry a temperature-sensitive *dnaZ* allele [*dnaZ*(Ts)2016] were examined. The temperature at which the *dnaZ*(Ts) protein becomes inactivated in vivo was investigated by measurements of deoxyribonucleic acid (DNA) synthesis at temperatures intermediate between permissive and nonpermissive. DNA synthesis inhibition was reversible by reducing the temperature of cultures from 42 to 30°C; DNA synthesis resumed immediately after temperature reduction and occurred even in the presence of chloramphenicol. Inasmuch as DNA synthesis could be resumed in the absence of protein synthesis, we concluded that the protein product of the *dnaZ* allele (Ts)2016 is renaturable. Cell division, also inhibited by 42°C incubation, resumed after temperature reduction, but the length of time required for resumption depended on the duration of the period at 42°C. Replicative synthesis of cellular DNA, examined in vitro in toluene-permeabilized cells, was temperature sensitive. Excision repair of ultraviolet light-induced DNA lesions was partially inhibited in *dnaZ*(Ts) cells at 42°C. The *dnaZ*⁺ product participated in the synthesis of both Okazaki piece (8-12S) and high-molecular-weight DNA. During incubation of *dnaZ*(Ts)(λ) lysogens at 42°C, prophage induction occurred, and progeny phage were produced during subsequent incubation at 30°C. The temperature sensitivity of both DNA synthesis and cell division in the *dnaZ*(Ts)2016 mutant was suppressed by high concentrations of sucrose, lactose, or NaCl. Incubation at 42°C was neither mutagenic nor antimutagenic for the *dnaZ*(Ts) mutant.

A *dnaZ*(Ts) mutant of *Escherichia coli* is inhibited in chromosome polymerization and cell division at 42°C (7). The *dnaZ* product also is required in vivo for all three stages of M13 and φX174 deoxyribonucleic acid (DNA) synthesis (10, 11) and for the growth of bacteriophage λ (34). *dnaZ* maps at min 10.5 on the recalibrated *E. coli* linkage map in the sequence *proC-tsx-dnaZ-purE* (37). The *dnaZ*(Ts) allele is recessive to the wild-type allele in partial diploid strains that carry F'13 (7).

Other *dna* genes include *dnaA*, -C, -I, and -P, which code for products that participate in the initiation of chromosome replication at its origin (1, 6, 17, 36). *dna* genes B, E (DNA polymerase III) (8, 22), G, and perhaps also C are essential for chromosome polymerization (3, 14, 38, 40).

DNA synthesis appears to proceed discontinuously on at least one (19, 23), if not both (18, 30), strands of the replicating chromosome. Replication via short intermediates requires additional initiation events after those that

take place at the origin of chromosome replication. Therefore, gene products that participate in chromosome duplication can function in initiation of replication at the origin, in initiation of a discontinuous DNA segment, or in the elongation of initiated DNA (or perhaps in several of these processes).

In vitro, purified *dnaZ* protein has no detectable DNA or ribonucleic acid (RNA) polymerizing activity in itself, although it has been shown to function with DNA polymerase III and elongation factors (EF) I and III in the conversion of RNA- and DNA-primed single-strand φX174, fd(M13), and ST-1(G4) DNA to duplex form (41). *dnaZ* product participates in a reaction sequence that leads to the binding of DNA polymerase III to primed templates and is dispensable for the DNA polymerization reaction after this binding (39). Thus the role of *dnaZ* in chromosome polymerization may be in initiation of DNA polymerization after priming.

In this paper, we report the physiological

effects of incubating a *dnaZ*(Ts) mutant [*dnaZ*(Ts)2016] at nonpermissive temperatures. The *dnaZ*(Ts)2016 protein is denatured gradually in vivo at temperatures of 37, 40, and 41°C and is renaturable after temperature refection. A return to 30°C, after a period of incubation at 42°C, permits *dnaZ*(Ts) cells to resume DNA synthesis at a rate greater than the normal 30°C rate. Using cellular DNA as template in toluene-treated *dnaZ*(Ts) cells, replicative DNA synthesis is inhibited at 42°C. Incubation of *dnaZ*(Ts) cells at 42°C inhibits the synthesis of both Okazaki piece and high-molecular-weight DNA and partially inhibits the ability of these cells to complete excision repair after ultraviolet (UV) irradiation. Incubation of a *dnaZ*(Ts)(λ⁺) lysogen at 42°C resulted in prophage induction and progeny phage production during subsequent growth at 30°C. There was no detectable mutagenic or antimutagenic effect of 42°C incubation on the *dnaZ*(Ts) mutant.

MATERIALS AND METHODS

Strains. AX727 [*F*-*lac gal dnaZ*(Ts)2016], H727 [*F*-*dnaZ*(Ts)2016 *thy rpsE tsx*], and AX729 (*F*-*lac gal dnaZ*⁺) have been described (7, 11). AX746 is a spontaneous *thyA* mutant of AX727, selected by the Caster technique (4); AX733 is a low-thymine-requiring spontaneous *deoB* or -*C* mutant of AX746. Temperature-insensitive (TS⁺) revertants were spontaneous mutants. *E. coli* C (from R. McKee) was used in the spheroplast transformation assay. Strain H5274 was obtained from D. S. Ray. Phage λ⁺ (wild type) was from A. D. Kaiser.

Growth conditions. The yeast extract-tryptone (YET) medium (15) contained 0.5% NaCl, and minimal medium base (15) was supplemented with glucose (10 mg/ml), thiamine hydrochloride (5 μg/ml), Casamino Acids (2.5 mg/ml), thymine (2 or 50 μg/ml), and L-valine (100 μg/ml) as required. PA agar and soft agar (9) were used for spheroplast transformation assays of φX174 DNA. Phage λ was titered in YET agar and soft agar containing 0.001 M MgSO₄. Temperature changes were accomplished by transfer of liquid cultures to previously equilibrated flasks.

Cell number, mass, and DNA synthesis determinations. Cell counts were made with a model ZB Coulter Counter after dilution in 0.9% NaCl-0.5% formaldehyde. Absorbance was measured in a Zeiss PMQII spectrophotometer at 540 nm. The steady-state synthesis of DNA was followed by the incorporation of [³H]thymine into 5% trichloroacetic acid-insoluble material.

In vitro DNA synthesis in toluene-treated cells. AX727 and AX727TS⁺2 cells were grown to 2 × 10⁸ cells/ml in YET broth, concentrated, and toluene treated to render them permeable. DNA synthesis in the permeable cells was followed by the incorporation of [³H]thymidine 5'-monophosphate into trichloroacetic acid-insoluble precipitates (21, 25). Cul-

tures of strains AX727 and AX727TS⁺2 were mixed with toluene at room temperature for 1 and 4 min, respectively.

Measurement of excision repair. Analysis of excision repair of UV-induced lesions followed the protocol of Youngs and Smith (44). Gradients were centrifuged at 20°C for 120 min at 30,000 rpm in an SW50.1 rotor.

Measurement of Okazaki piece synthesis. Ten-milliliter cultures were pulsed for 10 s with 40 μCi of [³H]thymidine (20 mCi/μmol) and analyzed by the procedure of Okazaki et al. (24), except that the pulse was stopped by cold pyridine-KCN-buffer (16), and the cells were lysed in 0.25 M NaOH and 0.01 M ethylenediaminetetraacetic acid. Lysates were mixed with φX174 single-strand DNA (0.36 μg) and centrifuged (24). The φX174 DNA (prepared from φX174h8ρ by the procedure of Brown and Dowell [2]) has a sedimentation coefficient of 16S in alkaline sucrose (29). Fractions of the gradient were collected and analyzed for trichloroacetic acid-insoluble [³H]thymidine and for the φX174 marker DNA in the spheroplast transformation assay (9).

Prophage induction experiments. Wild-type λ lysogens of AX727 and AX727TS⁺2 were grown at 30°C in YET broth containing 0.001 M MgSO₄ to 5 × 10⁷ cells/ml, and a portion of the culture was shifted to 42°C. After a return to 30°C, free phages were titered at 37°C on strain AX729.

Mutant frequency determination. Cultures incubated at 30 or 42°C were plated on selective media at 30°C. Strain AX727 and AX727TS⁺2 were plated on minimal medium containing glucose, thiamine hydrochloride, and valine to select valine-resistant mutants (20). Strains AX746 and AX746TS⁺14, both *thyA*, were plated on minimal medium containing glucose, thiamine hydrochloride, and 2 μg of thymine per ml to select low-thymine-requiring mutants (*deoB* and -*C*).

Chemicals. [³H]thymine and [³H]thymidine were obtained from New England Nuclear, [³H]thymidine triphosphate from Schwarz/Mann, nicotinamide adenine dinucleotide, dextran (average molecular weight of 10,400), and chloramphenicol from Sigma Chemical Co., and deoxyribonucleotides and adenosine 5'-triphosphate (ATP) from P-L Biochemicals.

RESULTS

DNA synthesis at intermediate temperatures. To determine the pattern of DNA synthesis at temperatures between permissive and nonpermissive, cultures of the thymine-requiring *dnaZ*(Ts) strain AX733 were shifted to 37, 41, and 42°C (Fig. 1). At 42°C, rate of DNA synthesis decreased immediately and, after an increase of 15% in total amount, degradation typically occurred. The extent of degradation observed in different experiments ranged from 0 to 30% of the DNA initially present. Degradation was observed consistently when the nonpermissive temperature was 43°C; about 30% of the DNA was solubilized (data not shown). At

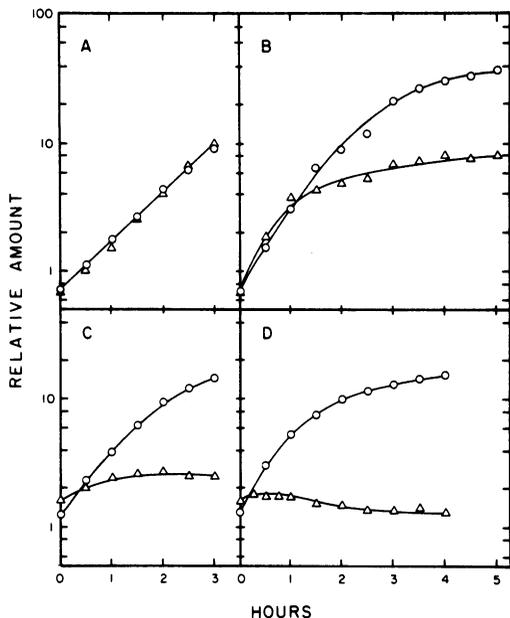


Fig. 1. Growth (O) and DNA synthesis (Δ) in the *dnaZ*(Ts) strain AX733 at 30 (A), 37 (B), 41 (C), and 42°C (D). Cultures were grown exponentially in YET broth containing [3 H]thymine (0.5 μ Ci per 2 μ g/ml). Relative amount 1 represents an absorbance of 0.1 and 1,000 cpm/ml.

41°C, rate of synthesis decreased immediately, the increment in total amount was 50%, and degradation usually did not occur. At 37°C, DNA synthesis proceeded at a rate greater than the 30°C rate for about two doublings; the rate then decreased gradually until the final amount of DNA was 10-fold the initial amount. The *dnaZ*(Ts)2016 protein appeared to be denatured gradually over a range of temperatures, rather than abruptly at a specific temperature.

Reversibility of DNA synthesis inhibition. A culture of the *dnaZ*(Ts) mutant AX733 was shifted to 42°C for 30 min and then back to 30°C (Fig. 2). A complex pattern of DNA synthesis and cell division ensued. DNA synthesis resumed immediately at a rate greater than the normal 30°C rate, then decreased to less than normal, and finally resumed the normal 30°C rate. Resumption of DNA synthesis at an abnormally high rate after temporary inhibition has been shown to result from premature reinitiation of chromosome replication (26). Cell division, which stops gradually during incubation at 42°C, resumed at 30°C, but the initial rate of division was less than the normal 30°C rate. These divisions were probably delayed by the requirement for completion of replication of those chromosomes that were inhibited by the

shift to 42°C (5, 13). Cell division next went into a rapid phase (rate greater than the 30°C rate) and finally, by the time the rapid DNA synthesis had reestablished the normal DNA/mass ratio (Fig. 2B) at 2 h after temperature reduction, cell division had resumed the 30°C, steady-state rate.

DNA synthesis resumed immediately on temperature reduction even when the incubation period at 42°C was 120 min and resumed even if the temperature was reduced in the presence of chloramphenicol (data not shown).

The thermosensitive *dnaZ*(Ts)2016 protein probably is reversibly inactivated by high temperature because (i) resumption of DNA synthesis occurred immediately on temperature reduction, rather than after a delay as would be expected if new protein synthesis were necessary, and (ii) DNA synthesis resumed in the presence of chloramphenicol.

DNA synthesis in vitro. The Moses and Richardson (21) procedure of treating cells with toluene to make them permeable to precursors was used to determine whether replicative DNA synthesis in the *dnaZ* mutant was temperature sensitive in vitro. In this procedure, replicative synthesis requires exogenous ATP, but repair synthesis can proceed in its absence (21). The wild-type revertant incorporated

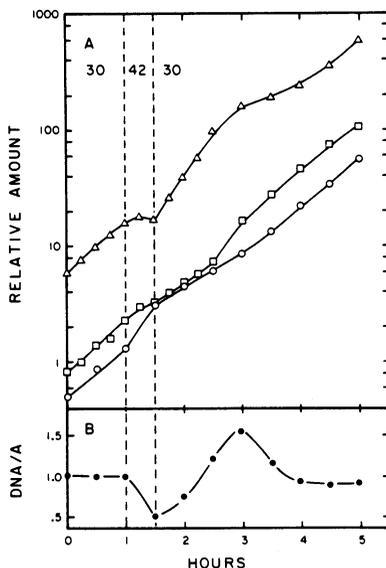


Fig. 2. (A) Growth (O), cell number (\square), and DNA synthesis (Δ) of strain AX733 [*dnaZ*(Ts)] during incubation in the sequence 30, 42, and 30°C. Incubation at 42°C was for 30 min. YET broth contained [3 H]thymine (0.5 μ Ci per 2 μ g/ml). Relative amount 1 represents an absorbance of 0.1, 10^7 cells/ml, and 100 cpm/ml. (B) Normalized DNA to mass ratio for the period covered in A.

[³H]thymidine 5'-monophosphate into DNA at both 25 and 42°C (Fig. 3A and B). At 42°C, the initial rate was twofold greater than the 25°C rate, but incorporation slowed after the first 10 min at 42°C. The *dnaZ*(Ts) mutant sustained only a limited amount of replicative DNA synthesis. Even at 25°C, a permissive temperature, ATP-dependent incorporation was only one-tenth of the revertant level (Fig. 3C). At 42°C, the level of incorporation was greater in the absence of ATP than in its presence (Fig. 3D). The amount of ATP-independent incorporation observed in the *dnaZ*(Ts) mutant at 42°C was approximately equal to that of the revertant culture.

UV-induced excision repair. Measurement of excision repair at 42°C could not be made in the *dnaZ*(Ts) strain AX727 because single-strand breaks occurred in DNA of both this strain and its TS⁺ revertants during incubation at 42°C even in the absence of UV irradiation. Consequently, the *dnaZ*(Ts) mutation was transferred by conjugation to strain H5274. Neither the *dnaZ*(Ts) recombinant, labeled H727, nor a TS⁺ revertant strain formed detectable single-strand breaks during growth at 42°C (in the absence of UV irradiation). The *dnaZ*(Ts) recombinant H727 and a TS⁺ revert-

ant were used for excision repair experiments.

dnaZ(Ts) and TS⁺ cultures were labeled with [³H]thymine, incubated for 30 min in buffer at 42°C to inactivate the *dnaZ*(Ts) product, irradiated with UV light, and incubated for 60 min in minimal medium at 42°C. Alkaline sucrose gradient analysis revealed that the *dnaZ*(Ts) culture was able to accomplish substantial excision repair at 42°C after exposures of 100 (data not shown) or 200 ergs/mm² (Fig. 4A); however

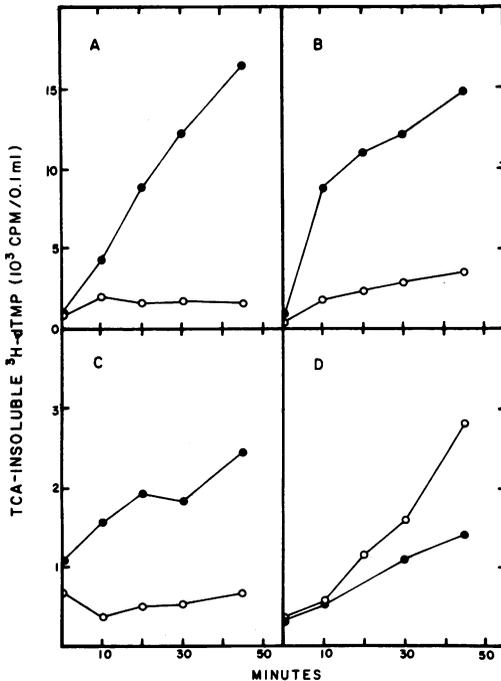


FIG. 3. Replicative (●) and repair (○) DNA replication *in vitro* in toluene-treated wild-type revertant AX727TS⁺2 (A, B) and AX727 [*dnaZ*(Ts)] (C, D) strains at 25°C (A, C) and 42°C (B, D).

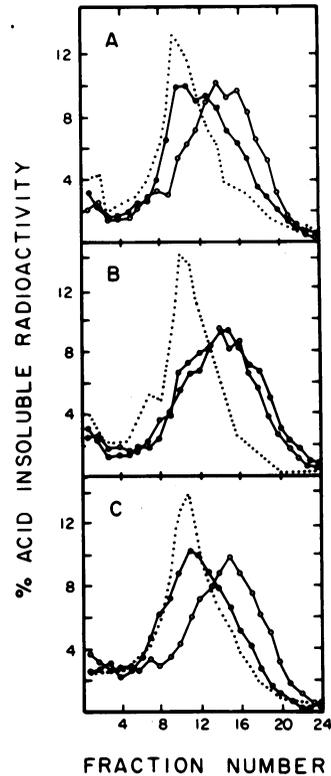


FIG. 4. UV excision repair in *dnaZ*(Ts) cultures. Cultures of H727 [*dnaZ*(Ts)] strain were grown in [³H]thymine-containing medium at 30°C, washed, resuspended in buffer, and incubated at either 30 or 42°C for 30 min. Cells incubated at 30°C were irradiated at room temperature, diluted into 30°C medium containing unlabeled thymine, and incubated at this temperature for 60 min in the dark. Cells incubated at 42°C were irradiated in a petri dish warmed to 42°C, followed by dilution and incubation at 42°C as above. Samples were lysed and sedimented through alkaline sucrose gradients. Sedimentation direction was from right to left. Symbols: (○) 2 min after UV irradiation; (●) 60 min after UV irradiation. The dotted curves represent unirradiated control cultures. The graphs display: (A) *dnaZ*(Ts), 200 ergs/mm², 42°C incubation; (B) *dnaZ*(Ts), 400 ergs/mm², 42°C incubation; (C) *dnaZ*(Ts), 400 ergs/mm², 30°C incubation. The total number of counts sedimented on each gradient ranged from 1,700 to 2,500.

there was only partial repair after an exposure to 300 ergs/mm² (data not shown) and no detectable repair after 400 ergs/mm² (Fig. 4B). The excision repair activity observed in the *dnaZ*(Ts) cultures after 200 ergs/mm² persisted even when the incubation at 42°C before irradiation was extended to 60 min (data not shown). A *dnaZ*(Ts) culture incubated at 30°C repaired the lesions after 400 ergs/mm² (Fig. 4C), as did a revertant culture exposed to 400 ergs/mm² and incubated at 42°C (data not shown). Thus, although the *dnaZ* product is not essential for excision repair after UV doses of 100 or 200 ergs/mm², it appears to play a role in a repair pathway that is required after exposure to more than 200 ergs of UV light per mm².

Polymerization of Okazaki pieces. Cultures of a *dnaZ*(Ts) mutant were incubated at 42°C for 3, 6, 12, 30, and 45 min and pulsed for 10 s with [³H]thymidine. As the period of incubation at 42°C increased, the amount of label incorporated into 8–12S and into high-molecular-weight DNA per 10-s pulse decreased concomitantly (Fig. 5, Table 1), except for the pulse administered after 45 min at 42°C. Perhaps partial degradation (Fig. 1) of existing DNA made available positions from which repair synthesis (i.e., short-piece synthesis) could begin. Alternatively, perhaps polymerization was so severely defective that short intermediates were not elongated rapidly enough to meet the ligation requirement that adjacent pieces be separated by only a missing phosphodiester bond.

The decrease in incorporation into both high-molecular-weight and Okazaki piece DNA is consistent with an initiation defect only if both daughter strands are synthesized discontinuously; it also is consistent with a role for *dnaZ*

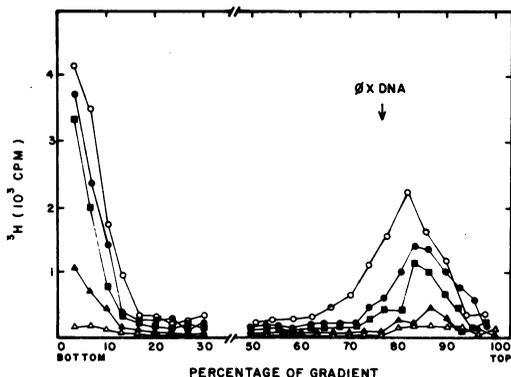


FIG. 5. Sedimentation of [³H]thymidine-labeled DNA extracted from strain AX727 [*dnaZ*(Ts)] after 10-s pulses after incubation at 42°C for 3 (○), 6 (●), 12 (■), 30 (▲), or 45 (△) min. The top 50% of the gradient fractions was normalized in position with respect to the ϕ X174 internal marker DNA.

TABLE 1. Synthesis of Okazaki pieces and high-molecular-weight DNA by the *dnaZ*(Ts) mutant at 42°C

Min at 42°C	cpm		% of total cpm	
	Large pieces ^a	Okazaki pieces ^a	Large pieces ^a	Okazaki pieces ^a
3	9,570	7,700	55	45
6	7,380	5,760	56	44
12	6,190	3,940	61	39
30	2,080	1,400	60	40
45	305	1,070	22	78

^a 0 to 20% of the gradient.

^b 60 to 99.5% of the gradient.

protein in polymerization per se in vitro.

Prophage induction during incubation at 42°C. Phage λ lysogens of *dnaZ*(Ts) and revertant strains were tested for prophage induction during 42°C incubation. During initial growth at 30°C, the free-phage titers were about the same in both cultures, approximately 1.5×10^{-3} phages/cell. The TS⁺ revertant culture was not induced when grown at 42°C continuously or when shifted to 30°C after 60 min of incubation at 42°C; free phages were spontaneously liberated at the same level as during growth at 30°C. The *dnaZ*(Ts) culture, on the other hand, was induced by temporary incubation at 42°C (Fig. 6). A 30-min period at 42°C was adequate for induction; the optimum period was 60 to 90 min. Continuous incubation of a *dnaZ*(Ts) lysogen at 42°C did not result in active progeny phage production; *dnaZ* product is essential for λ growth (34).

Suppression of DNA synthesis inhibition by increased osmotic pressure. Addition of sucrose or lactose (0.45 M or 16%) to minimal medium or of NaCl (0.5 M) to YET broth suppressed the temperature sensitivity of DNA synthesis, growth, and cell division of a *dnaZ*(Ts) mutant. Dextran (16%) and glycerol (2 M) failed to suppress the temperature-sensitive phenotype (data not shown).

Test for mutagenicity of incubation of *dnaZ*(Ts) mutants at 42°C. There was no evidence of mutator or antimutator activity during 120 min of incubation at 42°C.

DISCUSSION

The *dnaZ* protein has been shown previously to be required in vivo for chromosome elongation (7) and for synthesis of M13 and ϕ X174 parental replicative-form (RF) DNA, RF DNA replication, and single-strand DNA synthesis (10, 11) and in vitro for synthesis of duplex DNA from primed single-strand M13(fd), ϕ X174, and G4(ST-1) templates (39, 41). In vivo, the

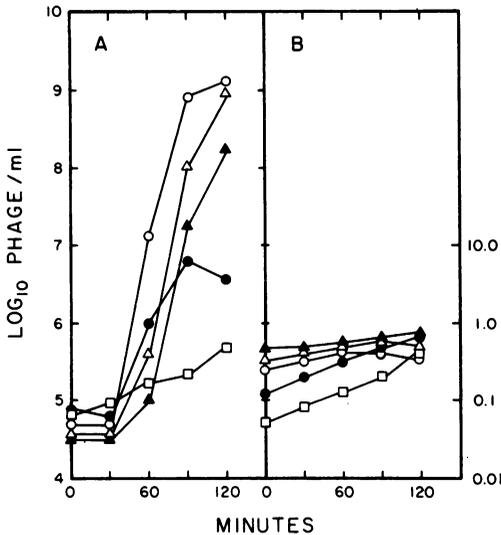


FIG. 6. Phage λ titer (A) and absorbance (B) of *dnaZ(Ts)* AX727(λ^+) cultures during 30°C incubation after 0 (\square), 30 (\bullet), 60 (\circ), 90 (Δ), and 120 (\blacktriangle) min of incubation at 42°C.

*dnaZ(Ts)*2016 protein is denatured gradually at 37 and 41°C. Once denatured at a nonpermissive temperature, it is renatured on lowering the temperature. The temperature-sensitive phenotype of the *dnaZ(Ts)*2016 strain can be suppressed by the addition of sucrose, lactose, or NaCl to the culture medium, presumably by stabilization of the missense *dnaZ* protein (12, 27). This suppression is not due to increased viscosity because the osmotically inert dextran (16%) does not suppress and the viscosity coefficient of a 16% dextran solution is 2.4 times that of a 16% solution of lactose.

Temporary incubation of a *dnaZ(Ts)* (λ) lysogen at 42°C results in prophage induction and, during subsequent incubation at 30°C, progeny phages are replicated. This result confirms and extends the observation of Taketo (33) on induction of a *dnaZ(Ts)* lysogen. If the model that single-strand breaks in DNA are responsible for prophage induction (31) is correct, the *dnaZ* mutants *B*, *E*, *G*, and *Z* (i.e., all the chromosome polymerization mutants), all of which are induced when lysogens are incubated at nonpermissive temperatures (28, 33), must be subject to single-strand nicks in DNA during incubation at high temperatures.

From *in vitro* work, the *dnaZ* protein, along with EF III, causes the transfer of EF I to primed phage DNA templates. DNA polymerase III then binds to the EF I-primed template complex and begins polymerization (39, 41). The *dnaZ* function is, therefore, in initiation

after primer synthesis. The exact mechanism of cellular chromosome synthesis *in vivo* still is unclear. Discontinuous synthesis of only one daughter strand (19) or of both daughter strands (18, 30) has been reported. Also, some Okazaki pieces might be repair intermediates derived from the excision of uracil-containing nucleotides from newly synthesized DNA (35). However, the finding that synthesis of both Okazaki piece and high-molecular-weight DNA are equally inhibited in *dnaZ(Ts)* (*polA*⁺) cultures is a strong indication that both chromosomal DNA strands are synthesized discontinuously.

The *dnaZ* product is not absolutely required for excision repair. A *dnaZ(Ts)* mutant incubated at 42°C could repair essentially all chromosomal lesions resulting from UV exposures of 200 ergs/mm² but not 400 ergs/mm². There are at least two pathways of excision repair after UV irradiation: a "short-patch" type, which appears to be catalyzed predominantly by DNA polymerase I (*polA* gene product), and a "long-patch" type, the requirements of which include the *rec* and *lexA* (*exrA*) gene products and probably DNA polymerase III (32, 43, 44, 45). The excision repair deficiency of the *dnaZ(Ts)* strain at doses over 200 ergs/mm² is comparable to that observed in a mutant of the "long-patch" pathway (*lexA* [*exrA*]) (44). Thus, the data suggest that *in vivo*, as *in vitro* (41), DNA polymerase III cannot effect extensive nucleotide incorporation in the absence of *dnaZ* protein.

The finding that some repair synthesis can take place in the absence of an active *dnaZ* product supports the *in vitro* observation (41) that *dnaZ* protein is not required by DNA polymerase I. This is a surprising result because, in the absence of a functional *dnaZ* protein, neither M13 nor ϕ X174 single-strand DNA is converted *in vivo* to parental replicative form (10), a reaction thought to be catalyzed by either DNA polymerase I or III (for a summary, see reference 10). Apparently, *dnaZ* protein is required *in vivo* by DNA polymerase I (and III) for parental RF synthesis but not for DNA polymerase I-catalyzed short-patch repair. Perhaps the reason for the difference in these reactions is that the single strand-to-RF conversion requires a priming event, whereas short-gap repair does not. It has been reported that an early displacement of the enzyme system responsible for RNA priming is not spontaneous and that DNA polymerase III complex (containing elongation factors and *dnaZ* protein) can effect this displacement more readily than can DNA polymerase I (42). Thus, the *dnaZ* protein might

be required to displace primer-synthesizing systems from phage templates, presumably by transferring EF I to the template, as a prerequisite for polymerization by either DNA polymerase I or III.

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