ISOLATION AND CHARACTERIZATION OF *dnaX* AND *dnaY* TEMPERATURE-SENSITIVE MUTANTS OF *ESCHERICHIA COLI*

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ABSTRACT

Escherichia coli mutants with temperature-sensitive (ts) mutations in *dnaX* and *dnaY* genes have been isolated. Based on transduction by phage P1, *dnaX* and *Y* have been mapped at minutes 10.4–10.5 and 12.1, respectively, in the sequence *dnaX purE dnaY*. Both *dnaX*ts36 and *Y*ts10 are recessive to wild-type alleles present on episomes. F13 carries both *dnaX*+ and *Y*+; the shorter F210 carries *dnaY*+, but not *X*+. Lambda transducing phages that carry *dnaX*+ or *Y*+ have been isolated, and hybrid plasmids of Col E1 and *E. coli* DNA from the CLARKE and CARBON (1976) collection also carry portions of the *dnaX purE dnaY* region. Results obtained with the λ transducing phages and the hybrid plasmids suggest that *dnaX* is a different gene from the previously characterized *dnaZ* gene, which is also near minute 10.5.——The *dnaX*ts36 mutant, after a shift to 42°, stopped DNA synthesis gradually, and the total amount of DNA increased two-fold. When this mutant was shifted to 44°, the rate of DNA synthesis dropped immediately and the final increment of DNA was only 10% of the initial amount. Replicative DNA synthesis in toluene-treated cells was completely inhibited at 42° and was partially inhibited even at 30°.——When the *dnaY*ts10 mutant was shifted to 42°, DNA synthesis gradually stopped, and the amount of DNA increased 3.6-fold. At 44°, residual DNA synthesis amounted to a two-fold increase. Replicative DNA synthesis *in vitro* in toluene-treated cells was inactivated after 20 minutes at 42° or by "preincubation" of cells at 42° before toluene treatment.—— The *dnaX* and *dnaY* products probably function in polymerization of DNA, although participation also in initiation cannot be excluded.

STUDIES of *Escherichia coli* chromosome replication have been facilitated by isolation and characterization of temperature-sensitive (ts) mutants that are defective in DNA synthesis at nonpermissive temperatures. DNA replication is known to involve the *dna* loci *dnaA, B, C, E, G, I, J, K, L, M, P,* and *Z* and *nalA, cou,* and *lig*. The products of *dnaA, B, C, I,* and *P* are necessary for initiation of DNA synthesis at the origin of replication (BEYERSMANN, MESSER and SCHLICHT 1974; FANGMAN and NOVICK 1968; KUEMPEL 1969; WADA and YURA 1974; ZYSKIND and SMITH 1977); *dnaB, C, E* (DNA polymerase III), *G,* and *Z* products are required for DNA polymerization (CARL 1970; FILIP *et al.* 1974; HIROTA, RYTER and JACOB 1968; WECHSLER and GROSS 1971; WICKNER and HURWITZ

1975; GEFTER *et al.* 1971; NUSSLEIN *et al.* 1971). DNA ligase and the 5′ → 3′ exonuclease activity of DNA polymerase I (*polA*ex) are essential for viability and for joining short pieces of DNA to high molecular weight strands (GOTTESMAN, HICKS and GELLERT 1973; KONRAD, MODRICH and LEHMAN 1973; KONRAD and LEHMAN 1974; OLIVERA and BONHOEFFER 1974). Functions of the other gene products in specific stages of replication have not been defined (FEISS, personal communication; SAITO and UCHIDA 1977; SEVASTOPOULOS, WEHR and GLASER 1977; RYAN 1976; BOURGUIGNON, LEVITT and STERNGLANZ 1973, GELLERT *et al.* 1977).

SEVASTOPOULOS, WEHR and GLASER (1977) reported the isolation of over 100 *E. coli* mutants that cease to synthesize DNA when shifted to the restrictive temperature. Two previously undiscovered loci were defined (*dnaL* and *dnaM*). One mutant, SG133, was thought to carry a single mutation that was 11% co-transducible with *purE*. This co-transduction frequency suggested that the ts mutation was in the *dnaZ* gene, which is located at min 10.5 and is 6.6% co-transducible with *purE* (WALKER *et al.* 1977). However, we report here that strain SG133 contains two ts mutations, each of which creates a defect specifically in DNA synthesis. Both mutations are co-transducible with *purE* and are located in previously unknown genes. These two genes are designated *dnaX* and *dnaY*.

In vivo and *in vitro* experiments with toluene-treated cells provide evidence that both *dnaX* and *Y* function in the polymerization phase of chromosome replication, rather than specifically in initiation of replication at the origin.

MATERIALS AND METHODS

Bacterial Strains: Table 1 is a list of strains used in this study. Information relevant to their

TABLE 1

Principal strains

Strain	Genotype	Characteristics	Source or reference
DG17	F⁻ *thi leu his arg met thyA deo lac mal xyl mtl rpsL* λ^R	Wild type but grew slowly on Yeast Extract-Tryptone (YET) without NaCl at 42°	SEVASTOPOULOS *et al.* (1977)
SG133	ts mutant of strain DG17	ts when plated at 42° on YET supplemented with 1% (or less) NaCl	SEVASTOPOULOS *et al.* (1977)
S9080	F⁻ *thi lac gal purE rpsL*	Wild type but grew slowly at 42° on YET without NaCl	M. MALAMY
GM241	F⁻ *thr leu pro purE uid his arg thi lac gal xyl mtl tonA rpsL*	Grew vigorously at 42° on YET without NaCl	B. BACHMANN
JH241	*thyA deo* derivative of strain GM241		

Strain	Genotype	Characteristics	Source or reference
GM36	*dnaX*ts36 transductant of strain GM241	ts on YET without NaCl; temperature-sensitivity was suppressed by 0.25% NaCl	
JH36	*thyA deo* derivative of strain GM36		
GM10	*dnaY*ts10 transductant of strain GM241	ts on YET with 0.5% NaCl	
JH10	*thyA deo* derivative of strain GM10		
AX727	*purE*+ *dnaZ*ts2016 transductant of strain S9080		FILIP *et al.* (1974)
JH727	*recA* derivative of strain AX727		
W3747	F13 *lac*+ *lon*+ *tsx* *dnaX*+ *dnaZ*+ *purE*+ *dnaY*+/*thi met*		E. LEDERBERG
χ790	F210 *purE*+ *dnaY*+/ *his* T_3^R		
F152/ KL253	F152 *lip*+ *gal*+/*thi* *pyrD trp tyr recA* *mtl xyl mal gal rpsL*		
JH1	*dnaX*ts *purE*+ *dnaY*ts transductant of strain S9080		
JH2	Spontaneous *tsx* mutant of strain S9080		
JA200	F+ *recA*/pLC*		C. PICKETT

* pLC numbers 6–2, 8–25, 10–24, 10–26, 22–1, 22–2, 22–8, 30–3, 30–4, 30–23 and 75 were used.

growth properties also is included, because it was observed that some wild-type K12 strains grew poorly at 42° when plated on medium with no added NaCl. Two such strains that grew poorly at 42° were DG17, the parent of SG133, and S9080. Ability of wild-type strains to grow at 42° on medium with no added NaCl was important because the residual amount of DNA synthesis in strain SG133 at the nonpermissive temperature was proportional to the NaCl concentration in the medium. This suggested that one or both of the mutations in strain SG133 was suppressible by NaCl.

Low-thymine requiring, spontaneous mutants of GM241, GM36 and GM10 were selected in two steps: *thyA* mutants were selected by the technique of CASTER (1967), except that trimethoprim was substituted for aminopterin, and then *deo* mutants were selected by spreading the *thyA* mutants on minimal medium supplemented with 2 μg per ml thymine.

A *recA* derivative (JH727) of strain AX727 was prepared as follows: Hfr JC5088 (*thyA*+

recA) was mated with a *thyA* mutant of strain AX727 and *thyA*+ recombinants were selected. These were screened for the *recA* character by testing their ability to support growth of λ*bio*10. One *thyA*+ *recA* derivative was labelled JH727.

Media: Yeast-extract tryptone medium (YET) (HOWARD-FLANDERS, SIMSON and THERIOT 1964) was used. It was supplemented with 0.5% NaCl unless otherwise specified. For phage λ growth, YET was also supplemented with 0.001 M $MgSO_4$. Minimal media (HOWARD-FLANDERS, SIMSON and THERIOT 1964) contained glucose (10 mg per ml), thiamine·HCl (5 μg per ml), amino acids (50 μg per ml), adenine (50 μg per ml) and thymine (2 or 50 μg per ml), as required. Bacteriophage P1*vir*a was grown in the medium described by ROSNER (1972), and P1 transductants were selected on the medium of CARO and BERG (1971).

Phage preparations: P1*vir*a was grown according to ROSNER (1972). λ*cI857dnaZ*+ and λ*cI857purE*+ transducing phages were grown according to WALKER, HENSON and LEE (1977). λ*cI857dnaZ*+18 is a plaque-forming phage and was grown lytically; other λ*cI857dnaZ*+ phages are defective and were induced by UV and heat from primary heterogenotes of genotype AX727*dnaZ*ts(λ+)(λ*cI857dnaZ*+). All λ*cI857purE*+ transducing phages are defective and were induced from primary heterogenotes of genotype S9080 *purE*(λ+)(λ*cI857purE*+). T6 was grown by infecting strain S9080 at a multiplicity of five.

P1 transduction and recombinant scoring: Transduction was performed according to WILLETTS, CLARK and LOW (1969). P1 was grown on the donor strain twice before use. Minimal medium plates, incubated at 30°, were used for selecting and scoring *purE*+. Temperature-insensitive (Ts+) recombinants were selected on YET plates supplemented with 0.1% NaCl and incubated at 44°; these conditions reduced background growth. Ts+ was scored, after the initial selection, on YET plates, without NaCl, incubated at 42°. T6 sensitivity or resistance among recombinants was scored by streaking strains on a YET plate with 10^9 phage suspended in a soft agar overlay.

Mating conditions for episome-containing strains: The mating procedure of MOODY and LUKIN (1970) was followed. Partial diploids were selected on minimal media containing streptomycin (200 μg per ml). There was no selection for F210 partial diploid strains. These mating mixtures were plated at 30° on YET medium supplemented with streptomycin, and males were identified by their ability to transfer *purE*+ and by sensitivity to M13 phage.

Episome curing: The procedure of HIROTA (1960) was used, except that cells were grown in YET broth (pH 7.6) with 100 μg per ml of acridine orange.

Spot test for transduction by λ. Transduction to Ts+ was performed according to the method of WALKER, HENSON and LEE (1977). Sterile lysates (0.05 ml) of λ*cI857dnaZ*+ or λ*cI857purE*+ phages were spotted on YET plates (0.1% NaCl) that had been spread with 2×10^7 recipient cells, either strain GM10(λ+) or GM36(λ+). The plates were incubated at 44° and positive transduction tests were recognized by the growth of several hundred colonies of the recipient strain. Transduction of the *dnaZ*ts strain AX727(λ+) was tested on YET plates with 0.25% NaCl, incubated at 42°.

Complementation by hybrid plasmids: The collection of hybrid colicin E1 plasmid-containing strains of CLARKE and CARBON (1976) were maintained on YET master plates spread with colicin E1. This colony bank was screened by replica plating onto recipients that were *dnaX*ts, *Y*ts or *Z*ts to determine which plasmids carry the *X*, *Y* or *Z* regions. The recipients were spread on YET plates (without NaCl or with 0.5% NaCl for the *Z*ts recipient) containing streptomycin (200 μg per ml) and incubated at 42° after replica plating. Complementation was identified by growth of the recipient.

Growth, cell number, and DNA and RNA synthesis: Culture absorbance was measured in a Zeiss PMQII spectrophotometer at 540 nm. Cell counts were made in a model ZB Coulter Counter after dilution in 0.9% NaCl-0.5% formaldehyde. The steady state synthesis of DNA or RNA was followed by the incorporation of [^{3}H]thymine or [^{3}H]uracil into 5% trichloroacetic acid (TCA)-insoluble material. YET medium was supplemented with 1 μCi/2 μg per ml [*methyl*-^{3}H]-thymine or 1 μCi per 20 μg per ml [5-^{3}H]uracil for five or six generations before experiments began. DNA was also measured by the diphenylamine reaction (GILES and MYERS 1965).

DNA synthesis in vitro: DNA synthesis *in vitro* was measured in toluene-treated cells by the procedure of MOSES and RICHARDSON (1970). Cultures were grown in YET medium with no added NaCl at 30° to an absorbance of 0.5, harvested, and mixed with toluene at room temperature for three min. Cultures to be "pre-incubated" at 42° were grown at 30°, shifted to 42° for two hr, during which time the absorbance increased to 0.5, and then harvested. [*methyl*-^{3}H]-thymidine triphosphate incorporation in TCA-precipitable material was used as the measure of DNA synthesis.

RESULTS

Strain SG133 has at least two temperature-sensitive mutations: The mutant SG133, isolated from the parental strain DG17, synthesized an increment of 59% of DNA after a shift from 30° to 41° (SEVASTOPOULOS, WEHR and GLASER 1977). When strain SG133 was transduced to Ts$^+$ by P1 bacteriophage grown on a *purE* Ts$^+$ donor, 11% of the transductants were *purE* (SEVASTOPOULOS, WEHR and GLASER 1977).

We suspected that mutant SG133 contained two (or more) mutations, for two reasons: (1) "revertants" of strain SG133, selected for the ability to form colonies at 42°, did not grow as well as the parental strain DG17 at 42°, and (2) "Ts$^+$" transductants of mutant SG133, prepared with P1 grown on the wild-type strain S9080, were not as fully temperature-insensitive as the parental strain DG17. These results suggested that the "revertants" were revertant at one locus and mutant at a second, and that "Ts$^+$" transductants were transduced to wild type at one locus and were still mutant at a second.

To test the possibility that mutant SG133 contained two mutations, P1 was grown on this strain and used to transduce ts (along with *purE*$^+$) into the wild-type strain GM241, which grew well at 42° on YET medium without added NaCl. The ts transductants were examined for ability to form colonies at 42° on YET plates with 0 or 0.25% added NaCl. Residual DNA synthesis and mass increase at 42° in YET broth were also measured. Two phenotypically different classes of ts transductants were, indeed, detected (Table 2, cross 1).

Class I ts transductants were unable to form colonies at 42° on YET plates without added NaCl, but grew at 42° on YET plates with 0.25% NaCl. In liquid YET medium without added NaCl, these transductants synthesized a residual two-fold increase in DNA and an 18 to 20-fold increase in mass (after five hours). As will be shown below, Class I ts transductants are mutant in a gene we designate *dnaX*; strain GM36, a typical Class I ts derivative of strain GM241, was chosen for further study.

The second class (Class II) of ts transductants failed to form colonies at 42° on YET plates with 0 or 0.25% NaCl. After shifting to 42° in YET broth (with no added NaCl), Class II transductants increased DNA and mass by factors of 3.5 and seven to eight, respectively. Class II transductants are defective in a gene that we designate *dnaY*. Strain GM10, a typical Class II transductant of strain GM241, was selected for further work.

To test the possibility that strains GM36 (*dnaX*ts) and GM10 (*dnaY*ts) contained more than one mutation each, phage P1 was grown on these mutants and used to transduce *purE*$^+$ to wild-type strain GM241. The properties of all ts

TABLE 2

P1 transduction experiments

Cross	Donor	Recipient	Selected marker; (No. of transductants)	Unselected marker; (No. of transductants)	ts transductants Class I (*dnaX*ts)	ts transductants Class II (*dnaY*ts)
1	SG133	GM241	*purE*+	ts	2 of 58 tested	56 of 58 tested
	*dnaX*ts *purE*+ *dnaY*ts	*dnaX*+ *purE dnaY*+	(300)	(70)		
2	GM10	S9080	*purE*+	ts		62 of 62 tested
	purE+ *dnaY*ts	*purE dnaY*+	(200)	(62)		
3	SG133	S9080	*purE*+	ts		13 of 14 tested*
	*dnaX*ts *purE*+ *dnaY*ts	*dnaX*+ *purE dnaY*+	(386)	(51)		
4	S9080	GM36	Ts+	*purE*		
	dnaX+ *purE*	*dnaX*ts *purE*+	(200)	(10)		
5	JH2	GM36	Ts+	*purE*		
	tsx dnaX+ *purE*	*tsx*+ *dnaX*ts *purE*+	(157)	(9)		
				tsx		
				(2)		
6	S9080	GM10	Ts+	*purE*		
	purE dnaY+	*purE*+ *dnaY*ts	(200)	(94)		

* The remaining ts transductant was a *dnaX*ts *dnaY*ts double mutant; this strain is designated JH1.

purE$^+$ transductants were identical to those of the parent donor. When strain GM36 was the donor, all the ts transductants resembled the *dnaX*ts donor, GM36. When strain GM10 was the donor, all the ts transductants resembled the *dnaY*ts donor, GM10. These data are consistent with the conclusion that strains GM36 and GM10 contain only one ts mutation each. Strains GM36 and GM10 reverted to Ts$^+$ with frequencies of 1×10^{-9} and 2×10^{-8}, respectively, and these revertants grew as well as the parental wild-type strain, GM241.

The *dnaY*ts mutation could be transferred to another wild-type strain, S9080 (Table 2, cross 2), but it was not possible to detect *dnaX*ts transductants of this strain (no ts strains out of 400 *purE*$^+$ transductants). This resulted from the fact that the wild-type strain S9080 grew poorly on YET medium without NaCl at 42°, and the obligatory addition of NaCl suppressed the ts phenotype of *dnaX*ts mutants. It was also possible to transfer *dnaY*ts from the double mutant SG133 (*danX*ts *dnaY*ts) to strain S9080 (Table 2, cross 3). Among the ts transductants of strain S9080 prepared with P1 grown on strain SG133, one strain, JH1, was a double mutant (*dnaX*ts *dnaY*ts). This mutant was recognized by the fact that it synthesized, after shifting to 42° in YET broth (plus 0.25% NaCl), an increment in DNA of only 10%. Strain JH1 was shown to carry both *dnaX*ts and *dnaY*ts alleles by using it as a donor to transduce strain GM241 to *purE*$^+$; of 45 *purE*$^+$ transductants that were also ts, nine were *dnaX*ts and 36 were *dnaY*ts.

Position of dnaX *and* Y: To determine the *dnaX-purE* and *purE-dnaY* distances, the percent co-transduction of *purE* with each *dnaX* and *Y* was measured. The data of Table 2, cross 1, were considered inadequate for this purpose because *purE*$^+$ transductants that were ts formed very small colonies on the selection plates. Possibly, some ts *purE*$^+$ transductants were overlooked during testing of those recombinants.

Therefore, phage P1 was grown on a *dnaX*$^+$ *purE* donor and used to transduce a *dnaX*ts *purE*$^+$ recipient to *dnaX*$^+$. The *dnaX*$^+$ transductants then were screened for *purE*. *dnaX* and *purE* are 5.3% co-transducible (Table 2, average of data in crosses 4 and 5), which corresponds to a map distance of 1.3 min (Wu 1966). *purE* and *dnaY* are 47% co-transducible (Table 2, cross 6) or 0.44 min apart.

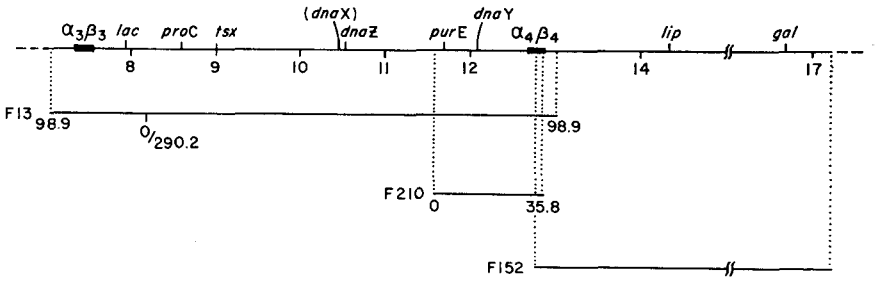

FIGURE 1.—Map of the *lac-gal* region of the *E. coli* chromosome (top; numbers refer to minutes) and of the bacterial sequences on F plasmids (numbers refer to kilobase pairs). The exact position of *dnaX* relative to *dnaZ* is unknown. Data taken from BACHMANN, LOW and TAYLOR (1976) and HU, OHTSUBO and DAVIDSON (1975). Not drawn to scale.

dnaX is located between *tsx* and *purE* (Figure 1). This was proved by cross 5 (Table 2), which demonstrated that *dnaX* is co-transducible with both *tsx*, located at min 9, and *purE*. *tsx* and *dnaX* are about 1.5 min apart (1.3% co-transducible by P1). *dnaX* is located near min 10.4–10.5, very near *dnaZ*.

The sequence of genes is *dnaX purE dnaY* (Figure 1) because the P1 transduction data prove that *dnaX* and *dnaY* must be on opposite sides of *purE*. *purE*$^+$ transductants that were ts and were prepared from P1 grown on a *dnaX*ts *purE*$^+$ *dnaY*ts donor were almost always (215 out of 216 tested) either *dnaX*ts *purE*$^+$ or *purE*$^+$ *dnaY*ts (Table 2, cross 1 and similar, additional experiments). Only 1 of the 216 such transductants carried both the *dnaX*ts and *Y*ts markers (Table 2, cross 3). Had *dnaY* been located between *dnaX* and *purE*, most *dnaX*ts *purE*$^+$ transductants would have been *dnaX*ts *dnaY*ts *purE*$^+$. Inasmuch as *dnaY* is 0.44 min from *purE*, *dnaY* must be near min 12.1 on the standard map.

Episome mapping: The *dnaX*ts and *dnaY*ts mutations are both recessive because they are complemented by the presence of their respective Ts$^+$ alleles. F13 contains both *dnaX*$^+$ and *Y*$^+$. All F13/GM36 *dnaXts* partial diploid strains were Ts$^+$; all F13/GM10 *dnaY*ts strains were Ts$^+$. On the other hand, F210 carries *dnaY*$^+$, but not *dnaX*$^+$. All F210/GM36 *dnaX*ts strains were ts, but F210/GM10 *dnaY*ts strains were Ts$^+$. F152 did not complement either mutation (Figure 1). All Ts$^+$ partial diploid strains, when cured of the episome by growth in acridine orange, became ts.

Mapping with specialized λ transducing phages: Walker, Henson and Lee (1977) described plaque-forming phages that carry the *dnaZ*$^+$ allele. These phages were isolated from a λ*cI*857 lysogen in which the prophage occupies a secondary attachment site near *purE*. Also isolated were defective λ*dnaZ*$^+$ and defective λ*purE*$^+$ phages (Irwin and Walker, unpublished data). Both the defective and nondefective λ*dnaZ*$^+$ phages formed with very low frequency [2×10^{-10} per plaque-forming unit (PFU)] and probably resulted from two events, such as a deletion followed by aberrant excision. Although all the λ*purE*$^+$ phages are defective, they form at a frequency of about 1×10^{-3} per PFU and presumably result from only one genetic event, *i.e.*, aberrant excision.

The use of these phages to complement *dnaX* and *Y* confirmed that *dnaX* and *Y* are separate and also indicated that *dnaX* is a gene distinguishable from *dnaZ* (Table 3). All six λ*dnaZ*$^+$ phages transduced Ts$^+$ to a *recA*$^+$ *dnaZ*ts (AX727) and to a *recA* *dnaZ*ts (JH727) recipient with comparable frequencies, which suggests that a complete *dnaZ*$^+$ allele is carried by these phages. Of the six λ*dnaZ*$^+$ phages, four carry *dnaX*$^+$, but two (λ*dnaZ*$^+$14 and λ*dnaZ*$^+$38) do not. This finding indicates the *dnaX* gene is separate and distinct from the *dnaZ* gene. The λ*purE*$^+$ phages carry *dnaY*$^+$, but not *X*$^+$ or *Z*$^+$. One phage (λ*dnaZ*$^+$20) was very unusual because it carried *dnaX*$^+$, *Y*$^+$ and *Z*$^+$. An alternative explanation is that it suppresses the *dnaY*ts mutation by an indirect mechanism rather than carrying all three wild-type genes.

Presence of dnaX *and* Y *on synthetic Col E1 hybrid plasmids*: Clarke and Carbon (1976) described the construction of a bank of clones containing Col E1 hybrid plasmids carrying *E. coli* chromosome segments. Several of these hybrid

TABLE 3

Transduction by specialized λ transducing phages*

Phage†	Recipient‡ GM36 *dnaX*ts	AX727 *dnaZ*ts	JH727 *recA dnaZ*ts	S9080 *purE*	GM10 *dnaY*ts
λ*dnaZ*+18	+	+	+	—	—
λ*dnaZ*+20	+	+	+	—	+
λ*dnaZ*+6	+	+	+	—	—
λ*dnaZ*+11	+	+	+	—	—
λ*dnaZ*+14	—	+	+	—	—
λ*dnaZ*+38	—	+	+	—	—
λ*purE*+11§	—	—	—	+	+

* +, positive transduction; —, no transduction.
† Phages were present in lysates with λ+ helper, except λ*dnaZ*+18, which is plaque-forming. Phages were named for the selection used to isolate them.
‡ All recipients were λ+ lysogens.
§ Eight additional, independently isolated phages were genetically identical to λ*purE*+11.

plasmids carry the *dnaZ* gene (CLARKE and CARBON 1976). The colony bank was screened by replica plating onto recipients that were *dnaX*ts or *Y*ts to determine if any plasmids carry these genes. Complementation of a *dnaZ*ts recipient also was tested. Six plasmids carried the wild-type *dnaZ* gene, but only four of these carry *dnaX*+ (Table 4). Another plasmid, pLC75, carries *dnaX*+, but not *dnaZ*+. These data corroborate the conclusion that *dnaX*+ and *dnaZ*+ are separate genes. In addition, one plasmid covers the *purE* region and three cover the *dnaY* region.

DNA synthesis inhibition at nonpermissive temperatures: To determine the pattern of growth, cell division, and macromolecular synthesis at nonpermissive temperatures, cultures of thymine-requiring *dnaX*ts and *Y*ts mutants were shifted from 30° to 42° or 44°. These measurements were made in YET medium without added NaCl because of the suppression of *dnaX*ts36 by as little as 0.25% added NaCl.

The temperature sensitivity of the *dnaX*ts mutant (strain JH36) resulted from

TABLE 4

Complementation of dnaX, Y *and* Z *by hybrid plasmids*

pLC	Recipient GM36 *dnaX*ts	AX727 *dnaZ*ts	S9080 *purE*	GM10 *dnaY*ts
75	+	—	—	—
6–2	+	+	—	—
30–3	+	+	—	—
30–4	+	+	—	—
30–23	+	+	—	—
10–24	—	+	—	—
10–26	—	+	—	—
8–25	—	—	+	—
22–1	—	—	—	+
22–2	—	—	—	+
22–8	—	—	—	+

a defect in DNA synthesis because, at 42°, DNA synthesis was inhibited, but RNA synthesis and growth continued (Figure 2). Residual DNA synthesis continued for about two hours, during which time there was a two-fold increase in amount of DNA. Why the residual synthesis took place in two steps in unclear. When the total amount of DNA was measured by the diphenylamine reaction, the residual synthesis was 1.8-fold (data not shown), confirming the experiment based on radioactive thymine incorporation. RNA synthesis and growth (measured by absorbance) proceeded faster at 42° than at 30° for about one hour; they then gradually slowed, but did not stop. After four hours, RNA had increased eight-fold and mass 13-fold. Cell division stopped gradually, also in a two-step process.

At 44°, the specificity of the defect in the *dnaX*ts mutant for DNA synthesis was dramatically demonstrated. DNA synthesis rate dropped immediately and the total increment was only 10% of the initial amount. RNA and mass increased by 500 and 700%, respectively, and residual cell division resulted in a doubling of cell number.

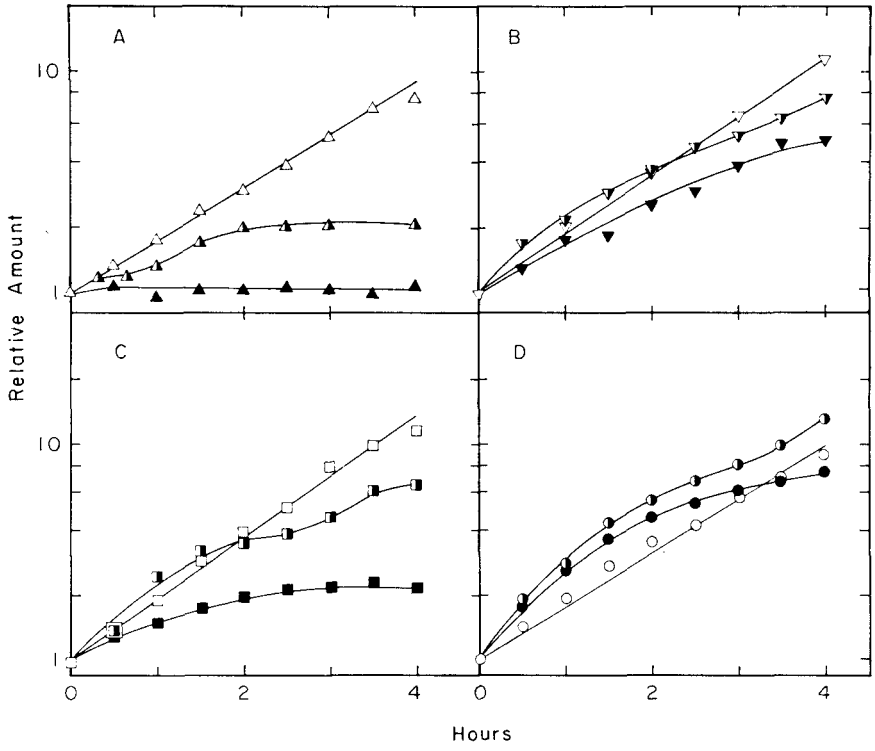

FIGURE 2.—DNA (A), RNA (B), cell number (C), and mass (D) increase in the *dnaX*ts mutant JH36 during incubation at 30° (open symbols), 42° (half-closed symbols), and 44° (closed symbols). (A) Relative amount 1 represents 4,000 cpm per ml TCA-insoluble [^{3}H]-thymine, (B) 4,700 cpm per ml TCA-insoluble [^{3}H]uracil, (C) 4×10^7 cells per ml (D) 0.07 A_{540}. The medium was YET with no added NaCl.

The gradual inhibition of DNA synthesis at 42° indicated that the *dnaX*ts36 mutation is leaky and made it impossible to determine if *dnaX* is involved in polymerization or initiation of replication. However, the pattern of inhibition of DNA synthesis at 44° includes an immediate decrease in rate of synthesis and a limited residual increase of only 10% the initial amount. These data are consistent with the conclusion that *dnaX* participates in polymerization, or perhaps in both initiation and polymerization of DNA.

Cultures of the *dnaYts* mutant were shifted to 42° or 44° for measurement of growth and macromolecular synthesis. At high temperature, DNA synthesis was specifically inhibited (Figure 3). At 42°, DNA synthesis was inhibited after three hours of incubation and the total amount of DNA increased by a factor of 3.6. Measurement of residual DNA synthesis by diphenylamine indicated a 3.5-fold increase in amount (data not shown). RNA and absorbance increased by factors of eight and seven, respectively. Cell division was gradually inhibited and the residual increase was five-fold. At 44°, DNA synthesis rate dropped immediately in the *dnaY*ts mutant and the total increase was two-fold; RNA and mass

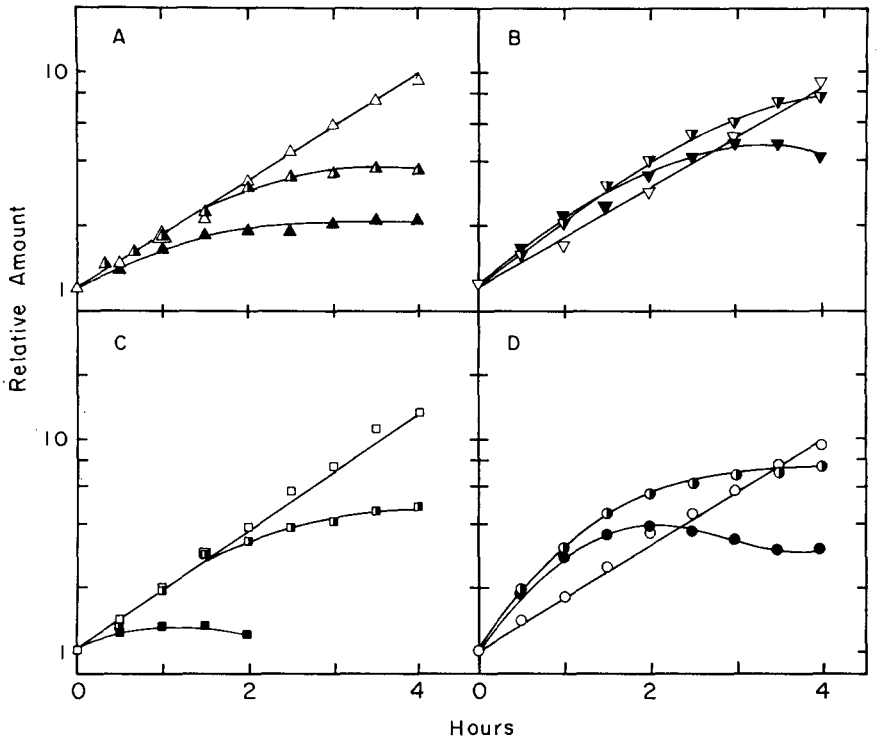

FIGURE 3.—(A) DNA, (B) RNA, (C) cell number, (D) mass increase in the *dnaY*ts mutant JH10 during incubation at 30° (open symbols), 42° (half-closed symbols), and 44° (closed symbols). (A) Relative amount 1 represents 3,700 cpm per ml TCA-insoluble [^{3}H]thymine, (B) 4,000 cpm per ml TCA-insoluble [^{3}H]uracil, (C) 3.7×10^7 cells per ml, (D) 0.076 A_{540}. The medium was YET with no added NaCl.

increased 4.6- and four-fold. Cell number increased only 30% and was not measured after lysis began at two hours.

The residual DNA increases of 3.5- and two-fold at 42° and 44° resulted presumably from leakiness of the *dnaY*ts10 mutation. Perhaps the *dnaY*ts10 product is inactivated slowly at these temperatures, or the functional *dnaY* product existing at the time of the temperature shift is diluted out by growth. This leakiness makes it impossible to determine from physiological experiments if *dnaY* has a polymerization or initiation function, although the immediate decrease in rate of synthesis at 44° suggests a polymerization defect.

Although many wild-type K12 strains did not grow well at 42° in YET medium with no added NaCl, the parental strain JH241, as well as revertants of JH10 and JH36, continued growth, macromolecular synthesis and cell division at 42° and 44° (data not shown).

Addition of 0.25% NaCl to YET broth completely suppressed the *dnaX*ts mutation and partially suppressed the *dnaY* mutation. When the thymine-requiring *dnaY*ts10 strain, JH10, was shifted from 30° to 42° in YET medium with 0.25% NaCl, the amount of DNA increased five-fold and mass increased 15-fold over a 3.5 hour period (data not shown).

dnaX*ts* dnaY*ts double mutants are more severely inhibited in DNA synthesis at 42° than is either single mutant*: Although the residual DNA synthesis at 42° in *dnaX*ts and *Y*ts mutants amounted to two- and 3.5-fold increases, respectively, *dnaX*ts *dnaY*ts double mutants synthesized increments of DNA of only 20 to 30%. Two such double mutants exist, strain SG133 (Sevastopoulos, Wehr and Glaser 1977) and JH1, a derivative of strain S9080. Residual DNA synthesis in strain SG133 at 42° was 25 to 30% of the initial amount, but mass increased to four times the initial amount (data not shown). In strain JH1, the residual DNA synthesis and mass increases were 10% and 300% of the initial amount (data not shown). In both cases, residual DNA synthesis was measured at 42° in YET medium with 0.25% NaCl because the parental strains of these organisms did not grow well at 42° in YET medium with no added NaCl. Unfortunately, isogenic strains that carry each one of the ts mutations are not available for comparison to strain SG133 or JH1. A *dnaX*ts derivative of strain S9080 was not detectable because the obligatory addition of NaCl to the medium suppressed the mutation. A *dnaY*ts transductant of strain S9080 (Table 2, cross 2) synthesized a 2.5-fold increment of DNA over the initial amount at 42° in YET broth plus 0.25% NaCl (data not shown).

DNA synthesis in vitro *in toluene-treated cells*: DNA synthesis in toluene-treated cells depends on polymerization at existing replication forks (Burger 1971). This procedure can be used to distinguish between defects in polymerization or initiation at the replication origin. Replicative synthesis was inhibited at 42° in a polymerization mutant, but proceeded at 42° in a ts initiation mutant until replication cycles in progress were completed (Mordoh, Hirota and Jacob 1970).

A culture of the *dnaX*ts mutant GM36 was grown at 30°, toluene-treated and assayed for DNA synthesis at 30° and 42° in the presence and absence of ATP.

By subtracting the level of repair synthesis (ATP-independent) from the level of synthesis in the presence of ATP, the extent of replicative synthesis can be determined (MOSES and RICHARDSON 1970). DNA synthesis in the toluene-treated *dnaX*ts cells at 42° was even more sensitive than in living cells in culture (Figure 4). Replicative DNA synthesis was completely inhibited at 42° and was partially inhibited even at 30°. These results with permeabilized cells indicated that *dnaX* is involved in polymerization and corroborate the conclusion drawn from *in vivo* measurements. For comparison, replicative DNA synthesis in toluene-treated cells of the wild-type strain GM241 was stable at 30° and 42° (Figure 5). Synthesis proceeded for at least 45 minutes at 42°, even after the cells had been "pre-incubated" at 42°.

Replicative DNA synthesis also was temperature sensitive in toluene-treated *dnaY*ts cells. Cells were grown at 30°, toluene-treated and assayed at 30° and 42° (Figure 6A, B). At 30°, replicative synthesis was normal. At 42°, replicative synthesis proceeded initially, but repair synthesis was stimulated. The initial replicative synthesis became temperature sensitive and was inhibited after 20 minutes, as determined by subtracting the synthesis due to repair from the total synthesis curve (Figure 6B). The replicative synthesis that occurred during the first 20 minutes at 42° was eliminated by "pre-incubation" of cells at 42° before toluene treatment and assay (Figure 6C). The inhibition of replicative synthesis after 20 minutes of incubation at 42° was demonstrated by a second procedure (Figure 6D). Cells were grown at 30°, toluene-treated and added to an assay mixture containing no [^{3}H]thymidine triphosphate; after 20 minutes, the labeled precursor was added. There was little, if any, replicative synthesis. These

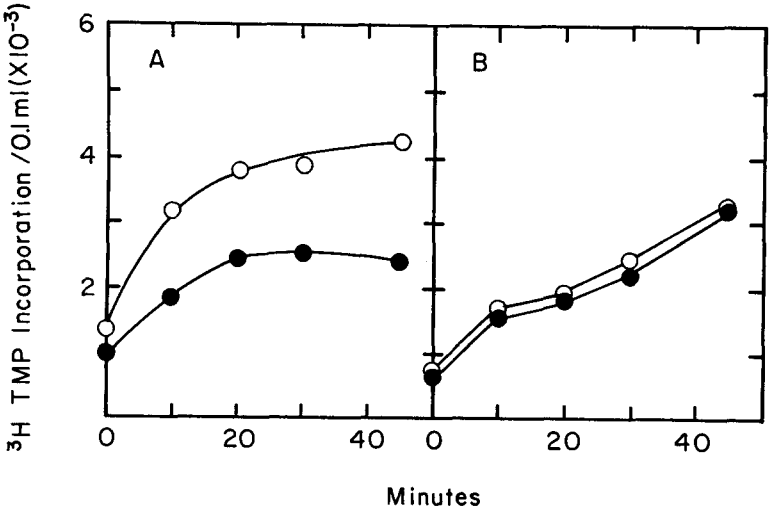

FIGURE 4.—Total (○) and repair (●) DNA synthesis in toluene-treated cells of strain GM36 (*dnaX*ts). A culture was grown at 30° in YET broth with no added NaCl, toluene-treated, and divided into two portions: (A) assay at 30°; (B) assay at 42°. "Total" synthesis was measured in the presence of ATP and includes replicative and ATP-independent repair synthesis.

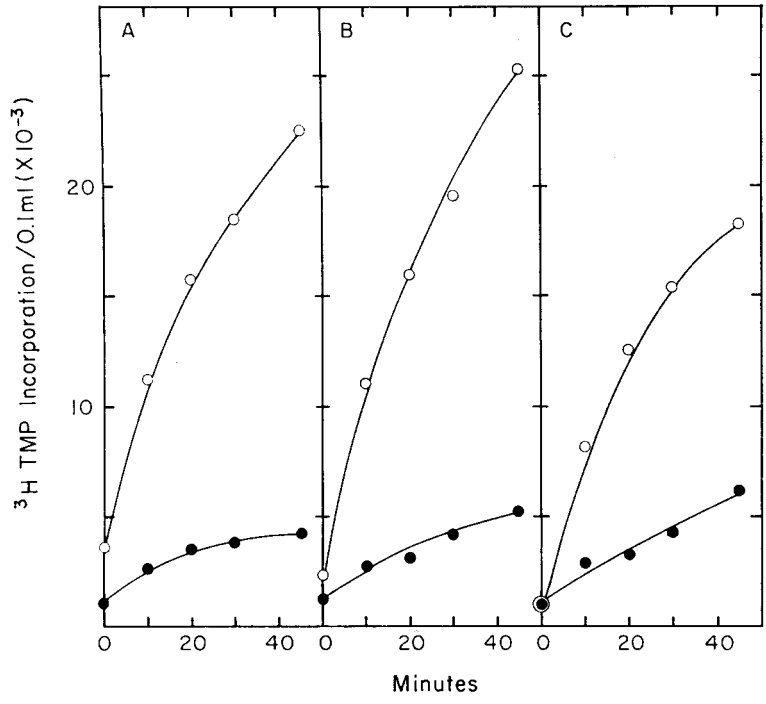

FIGURE 5.—Total (○) and repair (●) DNA synthesis in toluene-treated cells of wild-type strain GM241. A culture was grown in YET broth with no added NaCl at 30°, toluene-treated, and divided into two portions: (A) assay at 30°; (B) assay at 42°. (C) A second culture was grown at 30°, shifted to 42° for two hours' incubation, toluene-treated and assayed at 42°.

TABLE 5

Growth of λ *in* dnaX*ts and* Y*ts mutants*

Host	Pre-incubation at 42°	Progeny yield (PFU/input phage) 30°	41°	42°	Ratio 41°/30°	42°/30°
GM241 *dnaX*+ *dnaY*+	no*	120	160	33	1.3	0.25
GM36 *dnaX*ts	no*	110	110	13	1.0	0.08
GM10 *dnaY*ts	no*	230	70	2.9	0.3	0.01
GM241	yes†	240	250	23	1.04	0.095
GM36	yes†	260	50	20	0.19	0.076
GM10	yes†	120	30	0.83	0.25	0.0069

* Cultures were grown in YET broth plus 0.001 M $MgSO_4$, but with no added NaCl, to about 5×10^8 cells per ml, infected with λ*cI*857 at an MOI of 0.1 (five min adsorption period), and diluted 1:200 in the same medium at 30, 41, or 42°. Incubation was continued for two hr, after which chloroform was added and the progeny titered on strain GM241 at 37°.

† Cultures were grown as above to about 2×10^8 cell per ml, shifted to 42° for 90 min, infected with λ*cI*857 at 42° at an MOI of 0.1 (five min adsorption period), and diluted 1:200 in the same medium at 30, 41, or 42°. Progeny were titered as above.

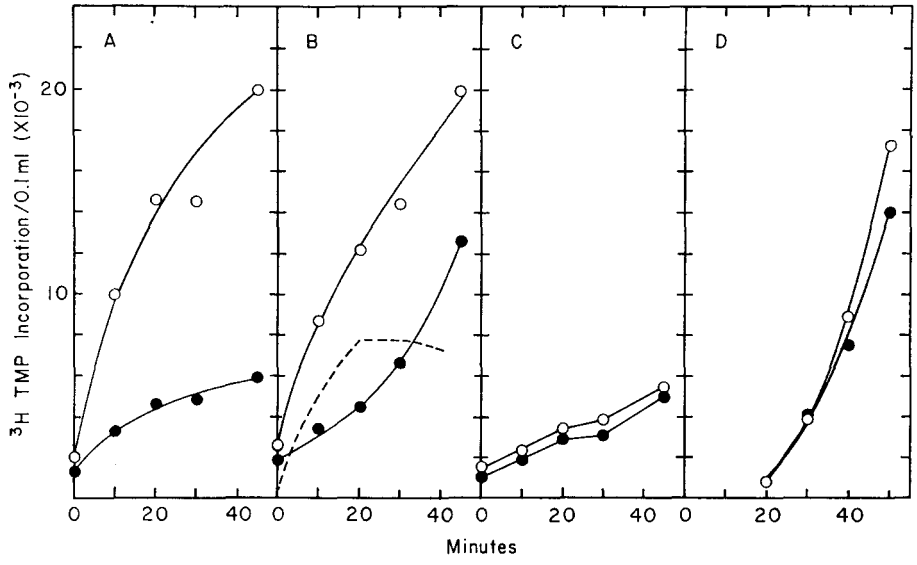

FIGURE 6.—Total (○) and repair (●) DNA synthesis in toluene-treated cells of strain GM10 (*dnaY*ts). A culture was grown in YET broth with no added NaCl at 30°, toluene-treated, and divided into two portions: (A) assay at 30°; (B) assay at 42°. In (B), the dashed curve represents replicative synthesis, *i.e.*, total synthesis minus repair synthesis. (C) A second culture was grown at 30°, "pre-incubated" at 42° for two hours, toluene-treated, and assayed at 42°. (D) A third culture was grown at 30°, toluene-treated, and added to an assay mixture complete with the exception that only unlabelled thymidine triphosphate was present. After 20 minutes, [^{3}H]thymidine triphosphate was added at the same specific activity as in other assays.

results are consistent with the interpretation that the *dnaY* product functions in polymerization.

In the case of both *dnaX* and *Y* mutants, these experiments with toluene-treated cells rule out the possibility that inhibition of DNA synthesis at high temperature is an indirect effect of precursor limitation, as would be expected if *dnaX* or *Y* were involved in precursor synthesis.

Growth of phage λ *requires the* dnaY *product*: Phage λ growth was only slightly restricted in a *dnaX*ts mutant at the nonpermissive condition, but was more inhibited at high temperature in a *dnaY*ts host. Cultures of wild-type and mutant strains were grown and infected at 30° and portions were then shifted to 41° and 42°. After two hours of incubation, progeny phage were titered. In medium without added NaCl, λ yield at 42°, even in the wild-type host, was reduced to about 25% of the yield at 30°. At 42°, the *dnaX*ts host produced about one-third the wild-type yield, but the *dnaY*ts mutant produced only about one-tenth the wild-type yield (Table 5).

When the hosts were pre-incubated at the nonpermissive temperatures and then infected, the *dnaX*ts host supported about the same degree of λ growth as did the wild-type strain. However, the *dnaY*ts host was limited in its ability to support λ growth and produced less than one progeny per infecting phage under these conditions.

The *dnaX* function, thought to be required for polymerization of the *E. coli* chromosome on the basis of experiments *in vivo* and in toluene-treated cells, apparently is not required for λ DNA polymerization. The *dnaY* mutant, apparently leaky in DNA synthesis at 42° and 44° *in vivo* but deficient in polymerization in toluene-treated cells, did not support λ growth. This finding suggests that *dnaY* functions in polymerization of DNA because all the known *dna*ts mutants that do not support λ growth (*dnaB*, *E*, *G*, and *Z*) are polymerization-defective strains (WICKNER 1978). Growth of phage M13 also requires the product of *dnaY*, but apparently not the product of *dnaX* (data not shown).

DISCUSSION

The *dnaX* and *Y* genes have been identified by separating two ts mutations from a mutant isolated by SEVASTOPOULOS, WEHR and GLASER (1977) after nitrosoguanidine mutagenesis. Both the ts mutations specifically cause defects in DNA synthesis as shown by the preferential inhibition of DNA synthesis at the nonpermissive temperature.

The *dnaX* gene is co-transducible with *tsx* and *purE* and is located near min 10.4–10.5, very near *dnaZ* (min 10.5). The genetic evidence that *dnaX* is a gene separate and distinct from the previously described *dnaZ* gene (FILIP *et al.* 1974) is based on transduction by λ*dnaZ*$^+$ phages and complementation by hybrid plasmids. Of six λ phages that carry a complete *dnaZ*$^+$ gene, only four transduced a *dnaX*ts recipient to Ts$^+$. In addition, two Col E1 hybrid plasmids were found to carry *dnaZ*$^+$, but not *dnaX*$^+$, and one hybrid plasmid was discovered to carry *dnaX*$^+$, but not *dnaZ*$^+$.

Based on P1 transduction data, *dnaY* has been located at min 12.1 on the clockwise side of *purE*. A possible sequence of the *dnaX dnaZ purE* and *dnaY* genes is shown in Figure 1, although the relative positions of *dnaX* and *Z* are not proved. The position of *dnaX* relative to other genes in this area (*popA*, *plsA*) is also unknown. The λ*dnaZ* phages cannot at this time be used to provide an unequivocal map of this region because they are thought to have been formed by two genetic events (WALKER, HENSON and LEE 1977).

It is possible to explain all the results presented here by assuming that the SEVASTOPOULOS, WEHR and GLASER 1977) mutant strain SG133 (*dnaX*ts *purE*$^+$ *dnaY*ts) carries only the two mutations *dnaX*ts and *Y*ts and that the strains GM36 (*dnaX*ts) and GM10 (*dnaY*ts) each contain only one mutation. However, it is also possible that strain SG133 carries three or more mutations and that there might be two (or more) mutations in either strain GM36 or GM10.

We also considered the possibility that the parental strain used by SEVASTOPOULOS, WEHR and GLASER (1977), DG17, might have carried either the *dnaX*ts or the *Y*ts mutations and that the mutation might not previously have been recognized because of its suppression by NaCl in culture media. However, this possibility was tested and strain DG17 does not harbor either of these mutations (data not shown).

The *dnaX*ts36 mutant is leaky at 42° and is suppressed by as little as 0.25% NaCl added to the enriched medium YET. However, DNA synthesis rate dropped immediately upon a shift to 44°, and residual DNA synthesis was only 10% more than the amount present at the time of the shift. The interpretation that *dnaX* is a polymerization gene is supported by the finding that DNA synthesis in toluene-treated *dnaX*ts cells is temperature-sensitive, without the need for "pre-incubation".

The *dnaY*ts10 mutant was leaky at 42° and 44° and was partially suppressed by adding NaCl to the medium. The *dnaY* product probably functions during polymerization because DNA synthesis in toluene-treated cells became temperature sensitive within 20 minutes of being incubated at 42°.

If *dnaX* and *Y* products function in polymerization, what might be their specific roles? WICKNER and HURWITZ (1976) reported that primed single strands of DNA supported polymerization on the addition of only four separately purified proteins—DNA polymerase III (the product of *dnaE*), the *dnaZ* gene protein, and Elongation Factors I and III. These Elongation Factors were purified from wild-type cell extracts, and the structural genes for these proteins are unknown. The *dnaY* product (required for λ and M13 growth) could correspond to one of the Elongation Factors. It seems unlikely that *dnaX* codes for one of the Elongation Factors because no requirements for *dnaX* could be demonstrated for M13 growth *in vivo*. However, the participation of *dnaX* in λ and M13 replication *in vivo* might not have been detected in the *dnaX*ts36 mutant because of leakiness or because NaCl suppressed the ts defect of this mutant.

MCHENRY and KORNBERG (1977) prepared a DNA polymerase holoenzyme that contained six (or more) proteins. The *dnaX* and *Y* genes could correspond to any of the four proteins for which genes are unknown. Another possibility is that *dnaX* codes for a product, as yet biochemically undefined, that is required for *E. coli*, but not λ or M13 replication. Finally, it should be pointed out that, although λ and M13 growth is inhibited in the *dnaY*ts mutant, it has not been proved that the growth inhibition is due to a defect in DNA synthesis. If the growth inhibition did reflect replication inhibition, the stage(s) of λ and M13 replication that requires the *dnaY* function is not known.

An interesting aspect of the *dnaX* and *Y* mutants is that double mutants (*dnaX*ts *dnaY*ts) were inhibited in DNA synthesis at 42° to a greater extent than either single mutant. This suggests that *X* and *Y* function in different reactions. Alternatively, *X* and *Y* products might function as a complex *in vivo*, the complex of *X*ts and *Y*ts products being even more unstable than a complex of wild-type and ts subunits.

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