THE CHARACTERISTICS AND GENETIC MAP LOCATION OF A TEMPERATURE SENSITIVE DNA MUTANT OF *E. COLI* K12

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ABSTRACT

A temperature sensitive strain of *E. coli* K12 has been isolated in which residual DNA synthesis occurs at the 40°C restrictive temperature; syntheses of RNA, protein and DNA precursors are not directly affected. The mutation has been designated *dna-325* and is located at 89 min on the *E. coli* map in the same region where the *dnaC* locus is found. *dnaC* mutants are considered to be defective in DNA initiation. Some of the data are consistent with the view that the *dna-325* mutation is temperature sensitive in the process of DNA initiation rather than DNA chain elongation: (1) more than two cell divisions occur after a shift to 40°C; (2) upon a shift down to 30°C, cell division occurs again only after the DNA content of the cells has doubled; (3) 80% more DNA is made at 30°C in the presence of chloramphenicol after prior inhibition of DNA synthesis at 40°C. These three observations indicate that rounds of DNA replication were completed at 40°C. Also (4) infective λ particles can be made at 40°C long after bacterial DNA replication has ceased. It appears however that some DNA initiation can occur at 40°C since (1) a limited amount of DNA synthesis does occur at 40°C after prior alignment of the chromosomes by amino acid starvation at 30°C, and (2) after incubation in bromouracil at the restrictive temperature, heavy DNA is found with both strands containing bromouracil.

TEMPERATURE sensitive mutants of DNA replication are being examined to investigate the many steps involved in DNA replication. The two major phenotypic classes found include those mutants which stop DNA synthesis immediately and those which carry out residual DNA synthesis upon a shift to the restrictive temperature. The former mutants could be defective in precursor synthesis or chain elongation. The latter class can be subdivided into initiation mutants and "leaky" mutants, in which initiation, precursor synthesis, and/or chain elongation continue for a while at the restrictive temperature. These *E. coli* mutants have been classified into seven groups (*dnaA–G*), six solely on the basis of their map location with a subdivision into two groups because of phenotypic differences of those mutants mapping near *leu* (*dnaC* and *dnaD*) (WECHSLER and GROSS 1971, GROSS 1972). Most of the groups are presumed to be involved in DNA chain elongation; it is known that *dnaE* codes for DNA polymerase III (GEFTER *et al.* 1971). *dnaA* (KUEMPEL 1969, HIROTA, MORDOH and JACOB 1970, ABE and TOMIZAWA 1971) and *dnaC* (CARL 1970, GROSS 1972) mutants are thought to be initiation mutants. This paper will describe a DNA mutant (*dna-*

325) which maps near *dnaC* and *dnaD* at 89 min on the *E. coli* genetic map but which differs in at least one characteristic from either *dnaC* or *dnaD* mutants. It has several characteristics in common with *dnaA* mutants and appears to be a leaky initiation mutant. However some of the criteria which have been used for defining an initiation mutant are ambiguous, and more stringent evidence is required to establish *dna-325* as a gene involved in DNA initiation.

MATERIALS AND METHODS

E. coli K12 bacterial strains used: Gene symbols are those used by TAYLOR (1970).

BW524, BW812, and BW824 originated from strain DG75; the other BW strains listed are derivatives of strain AT2459.

DG75, a W1485 F^- (from W. MESSER) derivative, F^-, *thyA*$^-$, *dra*$^-$, *leu*$^-$.

AT2459, *serB22*, *thi-1*, $(\lambda)^-$ (from A. L. TAYLOR).

BW524, derived by treatment of DG75 with nitrosoguanidine; this strain contains two temperature sensitive mutations, see MATERIALS AND METHODS, below.

BW812, a spontaneous streptomycin-resistant derivative of BW524.

BW824 (originally called BWd24), F^-, *thyA*$^-$, *dra*$^-$, *leu*$^-$, Str^r, *ampA*$^-$, *dna-325*, derived by a mating of BW812 × Hfr DG358.

BW825, *dra*$^-$, *thi*$^-$, *dna-325*. Derived by transducing AT2459 with P1·BW824, selecting for Ser^+ colonies and then temperature sensitivity. This strain is sensitive to thymidine, due to the *dra*$^-$ trait.

BW827, *thi*$^-$, *dna-325*. Derived from AT2459 and prepared like strain BW825, it is thymidine resistant since it is *dra*+.

BW828, *thi*$^-$, *dra*$^-$, *thyA*$^-$, *dna-325*. A low thymine-requiring derivative of BW825 obtained by trimethoprim treatment (STACEY and SIMSON 1965).

BW830, *thi*$^-$, *lysA*$^-$, *argA*$^-$, *thyA*$^-$, *dra*$^-$, *dna-325*. Derived from BW828 by selecting a Thy^+ transductant carrying *lysA*$^-$ and *argA*$^-$ and reisolating a Thy^- strain by trimethoprim treatment (STACEY and SIMSON 1965).

BW831, a spontaneous revertant of BW828 to temperature resistance, *thi*$^-$, *dra*$^-$, *thyA*$^-$, *dna-325*, (*sup*+?). See RESULTS.

BW832, *thi*$^-$, *dra*$^-$, *thyA*$^-$. A temperature resistant strain selected by transducing BW828 with P1 grown on cells wild-type for the *dna-325* trait.

BW833, a derivative of BW824, *recA1*, Nal^r, *ampA*$^-$, Str^r, *leu*$^-$, *thy*$^-$, *dna-325*. The *recA1* trait was introduced by mating BW824 with KL163 (from BROOKS LOW).

AB1157, *thr*$^-$, *leu*$^-$, *arg-3*, *his*$^-$, *pro*$^-$, *thi*$^-$, *sup*+, Str^r (from A. J. CLARK).

C600G, *thr*$^-$, *leu*$^-$, *thi*$^-$(?) (from M. MESELSON).

DG10, *leu*$^-$, *thr*$^-$(?) *lac*$^-$, (λ), Str^r, *thi*$^-$, *thy*$^-$. A derivative of AB313 (from A. J. CLARK).

DG358, Hfr *gal*$^-$, *thr*$^-$, *met*$^-$, *thy*$^-$, *ampA*$^-$, Str^s. A derivative of strain P7201 (from S. BRENNER) resistant to 200 μg penicillin/ml. The mating origin is near 77 min on the genetic map with direction of transfer: *argC* . . . *ampA* . . . *pyrB* . . . *leu*. . . .

DG360, *thi*$^-$. Derived from AT2459 by transduction with P1·BW824, selection for Ser^+ and then further selection for thymidine and temperature resistance. Essentially isogenic with strain BW827 except for the gene *dna-325*.

KLF1/AB2463 (from B. Low) F′ KLF1 (*thr*+, *ara*+, *leu*+, *serB*+)/AB2463 *thr*$^-$, *leu*$^-$, *proA*$^-$, *thi*$^-$, *his*$^-$, *argE*$^-$, *recA13*, $(\lambda)^-$, *lac*$^-$, Str^r, *gal*$^-$, *ara*$^-$, *xyl*$^-$, *mtl*$^-$.

WA993, F+, *hsp*$^-$($r^-_k m^-_k$), *met*$^-$, *gal*$^-$, *ara*$^-$(?). (From S. LINN).

X36, F^-, *argH*$^-$, *ura*$^-$, *try*$^-$, *his*$^-$, *purC*$^-$, *thi*$^-$, Str^r (From S. BRENNER).

Bacteriophage: P1 vir a (from S. BRENNER), called P1 in this text, was used in the transduction experiments. λ·K (λ grown on K12 strain) and λ·O (grown on an $r^-_k m^-_k$ host), from S. LINN, were used for testing the *hsp* locus. T4 amber (*am*B22 and *am*E51), ochre (427) and umber (T4B *ac* qe L4P41) mutants were obtained from P. SCOTTI.

Media: Minimal medium consists of two solutions which are mixed after autoclaving (CLARK

and MAALØE 1967). Glucose was added to a final concentration of 0.2%. This medium was supplemented with 100 μg of DL-amino acids/ml, casamino acids (0.1% final concentration unless otherwise noted), 10 μg thymine/ml (unless otherwise noted) and/or 2 μg thiamine/ml as needed for growth of a particular strain.

Nutrient yeast agar (NYA) plates contain 2.3% Difco nutrient agar and 0.5% Difco yeast extract. When used in bacterial matings, 100 μg streptomycin/ml were added. L broth is the medium described by LURIA, ADAMS and TING (1960) with the $CsCl_2$ omitted. Tryptone plates consist of bacto-tryptone (1%), NaCl (0.8%), glucose (0.1%) and agar (1.5%). All other media and agar plates are as described in WOLF, NEWMAN, and GLASER (1968). Chloramphenicol was a gift of Parke Davis and Company.

Determination of cell numbers: Bacteria were counted by use of an electric particle counter employing a Tennelec preamplifier, a Tennelec linear amplifier, and a Nuclear Data pulse height analyzer.

Growth and filtration of cells: Bacteria from a stationary phase culture were inoculated into supplemented minimal medium to a final concentration of about 1×10^6/ml. The culture was placed in a shaker bath partially filled with ice; the bath was turned on automatically by a timer at a time appropriate for a given culture so that the cells were at the desired titer in the morning (7×10^7 to 2×10^8 depending upon the experiment). Culture media were changed by harvesting the bacteria on 0.22μ Millipore filters, washing with pre-warmed minimal medium (without glucose), and resuspending the bacteria in given fresh pre-warmed medium.

Radioactivity measurements: The *in vivo* incorporation of ^{3}H-thymine into DNA was measured by using the batch method described by BYFIELD and SCHERBAUM (1966). Biopette (Becton, Dickinson and Co., Orangeburg, N.Y.) disposable plastic tips were used for measuring 0.05 ml of culture onto Whatman 3MM filter paper discs. Following the treatment in cold trichloroacetic acid, the filters were washed once with iced water, then two times with boiling water followed by 95% ethanol. The dried discs were placed in 5 ml of diluted Packard Permafluor, a toluene based scintillation fluid, and counted in a scintillation counter.

Interrupted matings: Cultures were grown to about 2×10^8/ml in L broth, the temperature sensitive F^- strains at 30°C and the Hfr strains at 37°C. Matings were performed at 37°C using the procedure of HANE and WOOD (1969).

F′ matings: The recipient strain BW833 was grown to about 2×10^8/ml in L broth at 30°C. KLF1/AB2463 was grown at 37°C in minimal medium supplemented with proline, thiamine, histidine, and arginine, a medium which maintains the F′ in the population. The cultures were mixed (female: F′ = 10:1) and allowed to mate at 37°C for 30 min. Colonies selected for their ability to grow at 30°C in the absence of leucine were then scored for their ability to grow at the restrictive temperature.

Transduction: The preparation of P1 on host strains and the transduction procedure used are the same as described in WOLF, NEWMAN and GLASER (1968).

Growth of λ and P1 at the restrictive temperature: BW824 was grown at 30°C in minimal medium supplemented with casamino acids (1%), leucine and thymine. The cells were grown to about 8×10^7/ml and then transferred to 41°C for 2 hr. Aliquots then were removed for incubation without infection at 30°C and 41°C. To avoid cooling the culture below the restrictive temperature, λ (m.o.i. = 4) was added directly to a portion of the culture at 41°C. One fraction of the infected culture was used for the assay of phage; ^{3}H-thymine (1.25 μCi/ml) was then added to the other aliquots. Radioactive samples were taken for 2 hr and phage production was assayed during the same period using C600G plating bacteria.

Induction with mitomycin was done by growing BW824 (λ) and DG10 to about 1×10^8/ml at 30°C, shifting the cultures to 41°C for 2 hr, adding the mitomycin C (2 μg/ml) and assaying for phage production after 150 min at both 30°C and 41°C using C600G plating bacteria. Incubation of BW824(λ) at the restrictive temperature was not sufficient by itself to induce λ growth.

The experiments using P1 infecting phage were similar to those using λ except that the recipient cells were grown in L broth supplemented with thymine, and the restrictive temperature used was 40°C. ^{3}H-thymine (50 μCi/ml) was added to the broth. The cultures were incubated after phage infection for 210 min, and X36 was used as plating bacteria.

Scoring temperature resistance: NYA plates were used to monitor growth at the restrictive temperature. To obtain temperature-resistant recombinants either by conjugation or transduction, a 3 hr expression at 30°C was necessary before the plates were transferred to the restrictive temperature. In early experiments 41°C was used; subsequently it was found that the wild-type strain DG75 itself has slightly impaired DNA synthesis at 41°C, so the restrictive temperature was changed to 40°C.

Scoring unselected markers: Transductants were purified and then were inoculated into 0.5 ml of supplemented minimal medium or L broth in individual cavities of a micro-culture container (Eleasa, P.O. Box 1031, Milano, Italy). After incubation at 37°C, or 30°C when necessary, replica plating was accomplished by using a board containing stainless steel spikes which fit into the cavities. It was sometimes necessary to dilute the bacteria by first replica plating into a microculture container filled with sterile minimal medium and then using this to replicate onto the desired selective plates. Control strains were similarly inoculated into cavities.

The *hsp* trait was scored by replica plating onto tryptone plates which had been spread with about 1×10^4 λ·O or λ·K infective particles. The plates were incubated overnight at 37°C. In all cases plaques could be discerned in the circles of bacterial growth spotted on λ·K. When grown on λ·O, a particular colony was hsp^- when many plaques resulted or hsp^+ when no plaques were observed. In some cases the results were confirmed by cross streaking onto a tryptone plate the phage and liquid culture to be tested.

Scoring of the phenotypic expression dra^- made use of the fact that the growth of dra^- strains is inhibited by thymidine (AHMAD and PRITCHARD 1969). Minimal plates containing supplements necessary for the growth of the colonies to be tested were spread with 0.2 ml of a 0.25% thymidine solution and allowed to dry before being spotted with cultures. The plates were scored when good growth was obtained on the control plates without thymidine (18 to 40 hr at 30°C depending upon the strain); too long an incubation resulted in growth in all colonies. (This appears to be true in liquid cultures as well; thymidine seems to retard growth, not prevent it completely.)

Determination of nucleoside triphosphate pools: The experimental procedure was that of EDLIN and STENT (1969). Cultures were grown in $^{32}PO_4$ medium at 30°C, shifted to 41°C when the titer reached 10^8/ml, and aliquots were removed for analysis at 40 min intervals. Extraction of the nucleotides was done with 0.4 M perchloric acid, followed 30 min later with 0.24 M KOH and 0.05 M $KHCO_3$. After centrifugation the supernatants were analyzed by Dr. G. EDLIN using two dimensional thin layer chromatography.

DNA replication in toluene-treated cells: The methods used were essentially the same as those used by BURGER (1971) which in turn were based on the assay devised by MOSES and RICHARDSON (1970). Cultures were grown at 30°C a minimum of 6 generations in minimal medium supplemented with leucine and ^{14}C-thymine (0.055 μCi/ml). Portions of the cultures to be starved for leucine (for 180 or 240 min, with similar results) were filtered, washed, and resuspended in medium of the same specific activity of ^{14}C-thymine but lacking the amino acid. Aliquots were also transferred to 40°C for 3 or 4 hr of incubation. The cultures were harvested at a density of $1.5–2 \times 10^8$/ml (about 8×10^7 for amino acid starved cultures) by centrifugation and resuspended at about 5×10^9 cells/ml in iced 50 mM potassium phosphate buffer (pH 7.4).

The suspensions were shaken with 1% redistilled toluene for 5 min at 30°C and then diluted 5-fold by the addition of a mixture in 70 mM potassium phosphate buffer (pH 7.4) yielding 13 mM $MgCl_2$, 0.13 mM NAD (freshly prepared), 1.3 mM ATP, 33 μM (each) dATP, dGTP and dBUTP, and 20 μM ^{3}H-dCTP (10 μCi/ml). (dBUTP was a gift of Dr. R. BURGER.) Incubation at 30°C or 40°C was halted after 20 min by chilling. In the case of the 40°C-treated cultures, the cells were harvested at 40°C, resuspended in phosphate buffer pre-warmed to 40°C, and maintained at 40°C until the toluenization step. The cells were toluenized for 1 min at 40°C or 5 min at 30°C; equivalent results were obtained using either procedure. The suspensions were then incubated with the reaction mixture at 30°C or 40°C, as above. The chilled cells were washed and lysed (BURGER 1971) and the lysates (0.9 ml) were mixed with 9 ml of CsCl (density 1.82 g/cc) and centrifuged a minimum of 60 hr at 24°C in a Beckman type 50 rotor at 39,000 rpm, to resolve light, hybrid, and heavy DNA. Fractions (approximately 0.2 ml) were collected from

the bottom of the centrifuge tube directly into vials containing 5 ml of a 1:10 water:Aquasol (New England Nuclear) mixture and counted in a liquid scintillation counter.

DNA analysis in the analytical ultracentrifuge: After growth at the restrictive temperature in the presence of 20 μg 5-bromouracil (BU)/ml, cells were lysed and mixtures of lysates and CsCl (density 1.76 g/cc) were centrifuged for 20 hr at 44,770 rpm in the Beckman Model E analytical centrifuge (MESELSON and STAHL 1958).

Isolation of mutant containing dna-325: Thymine starvation at the restrictive temperature was used as a means of enriching the culture with those cells which could not continue DNA replication at that temperature and would therefore not undergo thymineless death. A culture of DG75 was grown in minimal medium supplemented with thymine and leucine to 2×10^8/ml and concentrated 10-fold in Tris maleic buffer pH 5 for mutagenic treatment (ADELBERG, MANDEL and CHEN 1965). The suspension was incubated in the presence of nitrosoguanidine (300 μg/ml) for 20 min at 37°C, freed of the mutagen by washing on a Millipore filter (0.22 μ) and then grown overnight at 30°C in minimal medium supplemented with casamino acids, thiamine and thymine. Then the culture was diluted into the same medium, grown to 1×10^8/ml at 30°C and Millipore filtered. The cells were suspended in minimal medium supplemented with casamino acids and were thymine-starved at 41°C for 240 min by which time the viable count decreased 100-fold. An aliquot was removed into medium containing thymine and grown overnight. A second thymine starvation was done at 41°C for 6 hr (with a 10-fold loss in viability) and colonies were then plated on NYA plates at 30°C and replica plated at 41°C to detect temperature sensitive mutants. Each mutant was then screened for the ability to synthesize DNA and protein by comparing the amount of ^{3}H-thymidine or ^{14}C-leucine incorporated into TCA precipitable material at 30°C *vs* 41°C at 10 min and again at 90 min. Many mutants were isolated, including strain BW524.

Preliminary data indicated that strain BW524 could continue to synthesize protein but not DNA at 41°C and in other respects met at least some of the criteria which were used for defining an initiation mutant. However, experiments with other temperature sensitive mutants intervened for close to a year, and when strain BW524 was reexamined, the strain not only could not make DNA at the high temperature, but protein synthesis was impaired as well. Since neither a spontaneous temperature resistant revertant nor a temperature resistant transductant could be isolated from BW524, it appeared likely that the strain carried two temperature sensitive mutations. This proved to be the case. One of the temperature sensitive mutations mapped near *pyrB* (68% co-transduction frequency–unpublished data); this mutation caused the cessation of both DNA and protein synthesis at 41°C and has not been investigated further. The second temperature sensitive mutation mapped within 3 min of *leu*. (Definitive mapping is shown below in RESULTS.) A strain containing this single temperature sensitive mutation was isolated and called BW824. The temperature sensitive mutation in strain BW824 was designated *dna-325*.

RESULTS

DNA replication and cell growth at the restrictive temperature: When a culture of strain BW824 growing in minimal medium was shifted to the restrictive temperature, both DNA replication and cell division continued for a while before stopping. An average 2.4-fold increase in cell number was observed; a typical result is shown in Figure 1. Since cell division depends upon termination of chromosomal replication and not initiation (HELMSTETTER and PIERUCCI 1968; CLARK 1968), a 2.4-fold increase in cell number at 40°C suggests that termination of chromosomal replication occurred in most cells. This value is consistent with the 2.6-fold increase in cell number found in an unsynchronized culture after completion of replicating chromosomes in a *dnaA* mutant (BEYERSMANN, SCHLICHT and SCHUSTER 1971). However, the generation time of the cells in the experiment was 140 min, and with such a slow growth rate a gap in DNA repli-

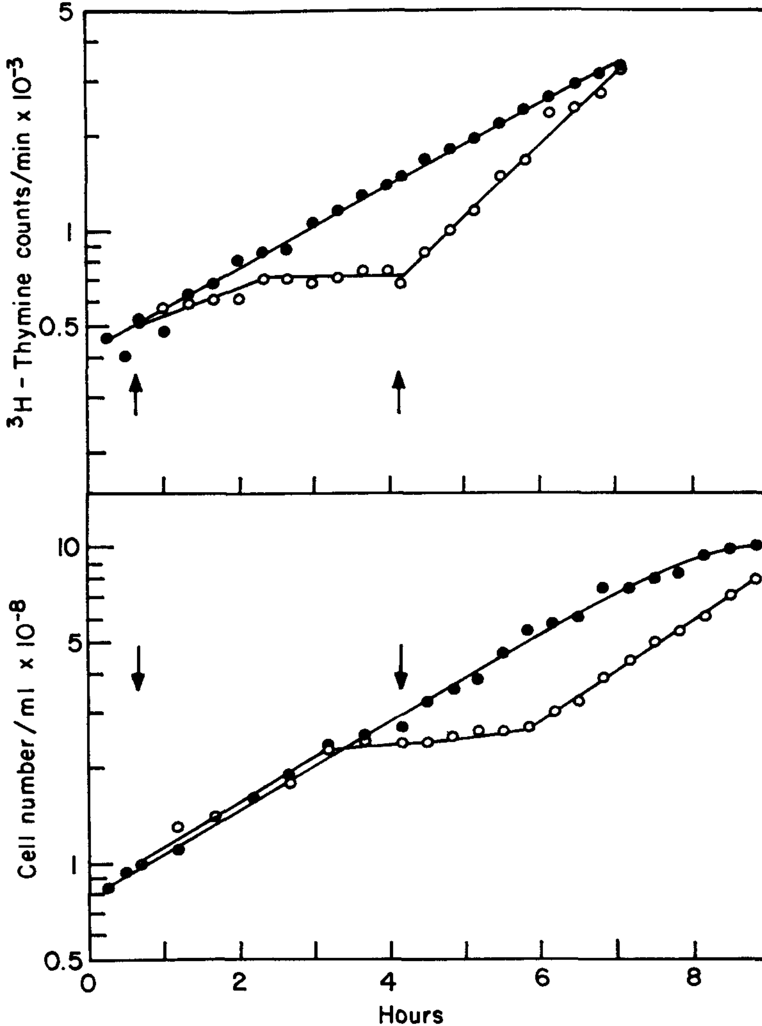

FIGURE 1.—DNA synthesis and cell division at 30°C and 40°C. BW824 was grown for over six generations in minimal medium supplemented with leucine and ^{3}H-thymine (5 μCi/ml). Then a portion of the culture was transferred to 40°C (O—O) at the time indicated by the first arrow and then shifted back to 30°C at the time indicated by the second arrow. The rest of the culture remained at 30°C throughout the experiment (●—●). Cell numbers and incorporation of radioactivity were measured as described in MATERIALS AND METHODS.

cation could occur somewhere in the division cycle (HELMSTETTER *et al.* 1968). Since it is not known where the gap occurs nor where new rounds of initiation occur in relation to the division cycle, it is difficult to interpret the significance of the 2.4-fold increase in cell number.

When the culture was transferred back to the permissive temperature, DNA replication resumed without a lag and at almost twice the rate as that of a culture that had been maintained at 30°C; cell division resumed after a doubling in the

DNA, and no cell synchrony was observed (Figure 1). A *dnaA* mutant acts in a similar manner (HIROTA, MORDOH and JACOB 1970).

RNA and gross protein synthesis were normal for at least 180 min of incubation at the restrictive temperature as determined by radioisotope uptake experiments (data not shown); however only 38% more DNA was made after the temperature shift (Figure 1). A total DNA increase of 39% is the theoretical value expected if initiation suddenly stopped in an exponentially growing culture in which there was only one replication fork per chromosome with the rate of synthesis constant throughout the division cycle (MAALØE and HANAWALT 1961). Obtaining a DNA increase of 38% in the experiment shown in Figure 1 was fortuitous, however, since the amount of DNA made at 40°C as measured by continuous ^{3}H- or ^{14}C-thymine labeling varied between 15 and 40% in different experiments. Results with overnight labeling of the cells continued to be inconsistent throughout the course of these experiments; compare the curve in Figure 1 to that in Figure 4 for the amount of DNA made at the restrictive temperature. When analyzed by 5-bromouracil (BU) labeling and CsCl density gradient ultracentrifugation, an average of 52% more DNA was made while at the restrictive temperature; the results of four such experiments are shown in Figure 2 A-D. Although it is difficult to quantitate, the existence of some heavy DNA is evident in at least two and perhaps three of the gradients indicating that some initiation can occur at the restrictive temperature. The small heavy peak seen in Figure 2C did not appear until about 130 min of incubation at 41°C, precluding the possibility that re-initiation took place very early after the temperature shift. The results of a more definitive experiment showing the existence of heavy DNA made at the restrictive temperature in strain BW824 are shown in Figure 3. Cells labeled overnight in medium containing ^{14}C-thymine were transferred to medium containing a mixture of BU and ^{3}H-thymine and were incubated at 40°C (Figure 3 A-C) or 30°C (Figure 3 D-F). At various times samples were removed, the cells were lysed, and the DNA was analyzed by CsCl density gradient ultracentrifugation. Although it is difficult to determine whether any heavy DNA existed after only 90 min incubation at 40°C (Figure 3A) due to the high tritium background in the sample, it is evident that heavy DNA was made in the 40°C culture after 180 min incubation (Figure 3 B, C). The majority of the ^{14}C-labeled DNA banded in the region of light density and remained unreplicated. By contrast, in the same period of time at the permissive temperature, almost all of the light DNA was replicated (Figure 3 D-F).

If the existence of heavy DNA is due to re-initiation, it is not known whether the initiation occurred at the origin or at random sites along the chromosome. Premature initiation at the origin ordinarily occurs in the presence of BU (ABE and TOMIZAWA 1967, WOLF, NEWMAN and GLASER 1968). These results could be interpreted in either of two ways: (1) the mutation allows initiations to occur, either at random chromosomal sites or at the origin, but only a limited amount of chain elongation occurs; or (2) the mutation blocks DNA initiation but the block is not complete and some chromosomes can re-initiate. It is possible that the existence of heavy DNA is not due to re-initiation of the chromosomes at the

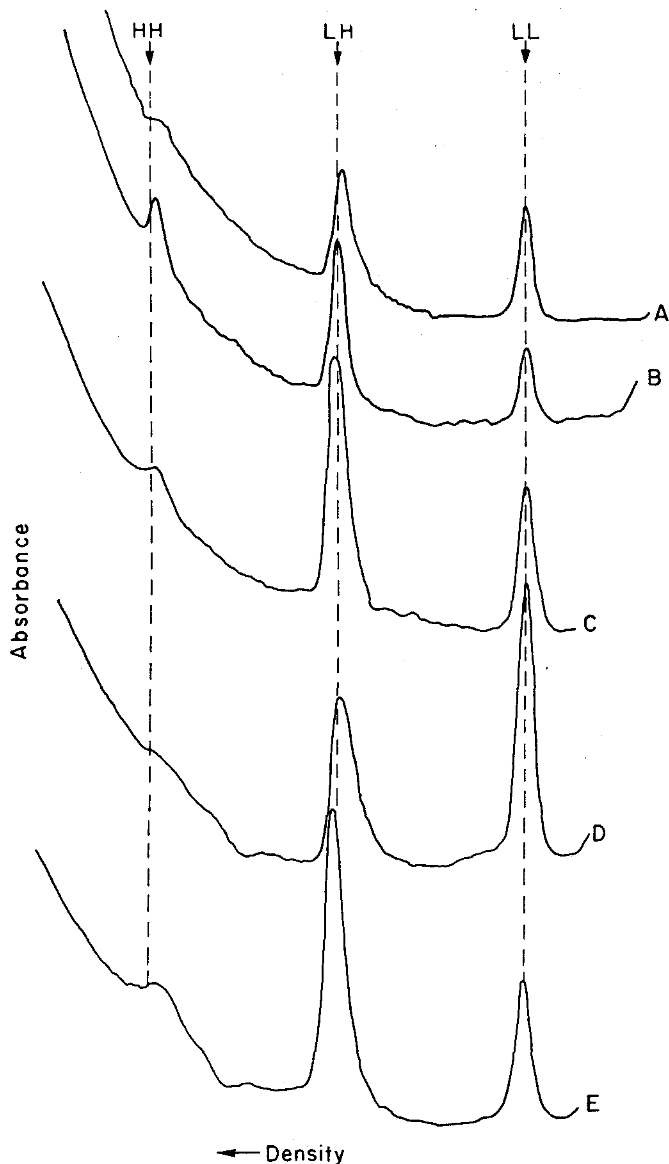

FIGURE 2.—Densitometer tracings of CsCl density gradients of DNA synthesized at 41°C in the presence of bromouracil. Cultures were grown exponentially in minimal media supplemented for BW824 with casamino acids, leucine and thymine (5 μg/ml), for BW828 with thiamine and thymine (5 μg/ml), and for BW830 with thiamine, lysine, arginine, and thymine (5 μg/ml). The approximate generation times in these media at 30°C were BW824, 90 min; BW828, 80 min; BW830, 90 min. The cultures were transferred to media containing no thymine and 20 μg BU/ml and simultaneously put at 41°C. After incubation the cells were lysed, placed in cesium chloride and the DNA examined after centrifugation for 20 hr at 44,770 rpm in a Beckman Model E ultracentrifuge. The procedures are described in MATERIALS and METHODS. The arrows show the expected positions for light (LL), hybrid (HL), and heavy (HH) DNA. A. BW824

restrictive temperature. The presence of three growing forks on the DNA at 30°C, all of which continued to replicate at 40°C in the presence of BU, would yield heavy DNA. This seems unlikely considering the long generation time of the cells (HELMSTETTER *et al.* 1968). Very active repair near the growing point occurring at the restrictive temperature would also result in heavy DNA. However, no apparent loss of radioactivity was observed when mutant cells were first grown for 2.5 generations in ^{3}H-thymidine and then filtered, washed and incubated in unlabeled medium at the restrictive temperature. Furthermore when at the restrictive temperature a culture was given a ^{3}H-thymidine pulse for 10 min and then chased with cold thymidine, no decrease in acid precipitable counts was seen for up to 140 min (data not shown). This indicates that the DNA which was made at 40°C was not repaired or hydrolyzed to any great extent at that temperature.

Resumption of DNA replication: The resumption of DNA replication at the permissive temperature does not require protein synthesis. Figure 4 shows the results of an experiment in which BW824 was incubated at 40°C for 185 min, 100 μg chloramphenicol/ml were then added to an aliquot at 40°C which 5 min later was placed at the permissive temperature. The amount of DNA made in the presence of chloramphenicol was about 80% of the preexisting DNA. (When the chloramphenicol was added 20 min prior to the shift to 30°C, approximately 50% more DNA was made; data not shown.) The significance of these amounts of DNA made will be dealt with in the DISCUSSION. Similar results were obtained with strain BW828 with 90 min preliminary incubation at 40°C. Since synthesis of the initiator protein is thought to be sensitive to chloramphenicol (LARK and RENGER 1969, WARD and GLASER 1969), the theoretical initiator protein must have accumulated during the 40°C incubation although perhaps in an inactive form which can be reversibly renatured at 30°C. *dnaA* mutants (ABE and TOMIZAWA 1971; BEYERSMANN, SCHLICHT and SCHUSTER 1971), a *dnaB* mutant (FANGMAN and NOVICK 1968) and *dnaC* mutants (P. CARL, personal communication; SCHUBACH, WHITMER and DAVERN, in press) also resume DNA synthesis in the presence of chloramphenicol after a prior incubation at the restrictive temperature.

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incubated at 41°C for 210 min. The amount of newly replicated DNA is approximately 44%. B. BW824 incubated at 41°C for 260 min. A similar experiment to that seen in A. The amount of newly replicated DNA is about 92% (54% hybrid and approximately 38% heavy). C. BW830 incubated at 41°C for 220 min. About 67% newly replicated DNA is seen (53% hybrid and approximately 15% heavy). D. BW828 incubated at 41°C for 240 min. 32% newly replicated DNA was observed. E. After 90 min incubation at 41°C an aliquot of the BW828 culture shown in D was transferred to 30°C for 20 min and then returned to 41°C, total incubation time was 240 min. About 95% new DNA replication is seen (59% hybrid and 36% heavy). The calculations of the amount of newly synthesized DNA took into consideration the fact that hybrid DNA contains one parental strand and therefore only half of it is newly replicated (PRITCHARD and LARK 1964); whereas heavy DNA is newly replicated in both strands. The percent newly replicated DNA is therefore the amount of BU-containing strands as a function of the amount of light-stranded DNA existing at the time of the shift to the restrictive temperature. The amount of heavy DNA present in the gradients obviously could only be roughly estimated.

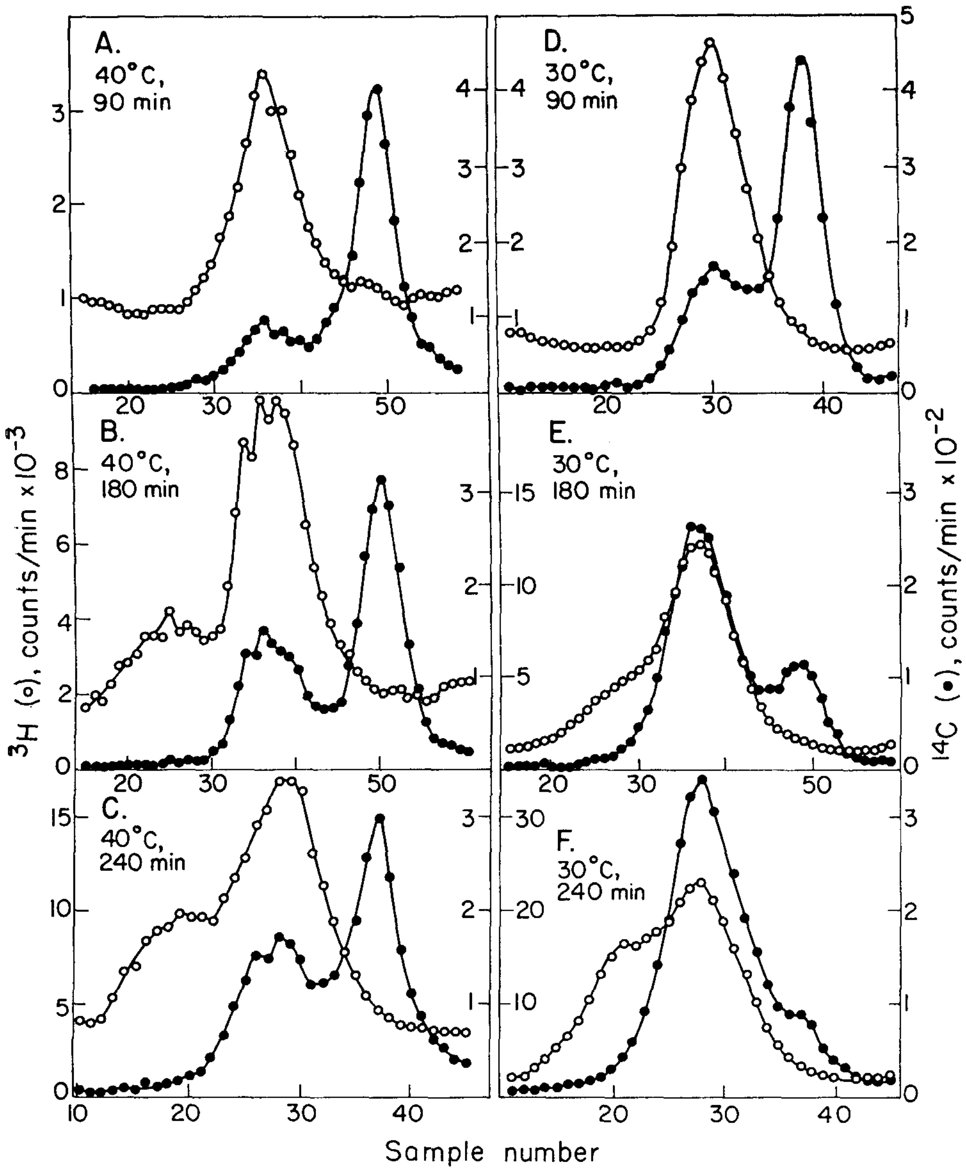

FIGURE 3.—CsCl density gradients of DNA made at 30°C *vs* 40°C after a simultaneous density shift. BW824 was grown at 30°C for over six generations in minimal medium supplemented with leucine and ^{14}C-thymine (0.055 μCi/ml). The cells were transferred to medium containing leucine, BU (20 μg/ml) and ^{3}H-thymine (1 μg/ml; 10 μCi/ml). Half the culture was incubated at 40°C for A. 90 min, B. 180 min or C. 240 min, and the other half at 30°C for D. 90 min, E. 180 min or F. 240 min. The cells were lysed etc. as described in MATERIALS AND METHODS, the only differences being that the cells were washed only once in pH 8.5 Tris-EDTA buffer and the gradient was formed at 42,000 rpm. The ^{3}H-thymine counts (O—O) are recorded on the left ordinate of each graph and the ^{14}C-thymine (●—●) on the right.

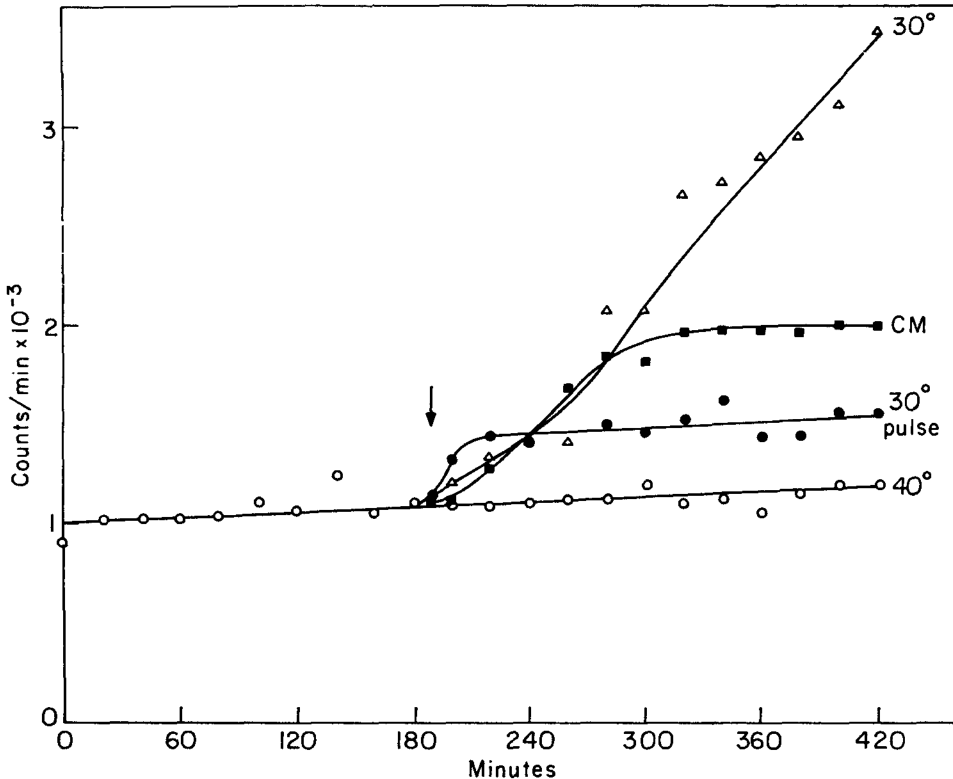

FIGURE 4.—The effect of chloramphenicol or a 30°C pulse on resumed DNA replication. Strain BW824 was grown for over six generations in minimal medium supplemented with leucine and ^{3}H-thymine (10 μCi/ml). The culture was transferred to 40°C at 0 min. Part of the culture remained at 40°C throughout the experiment (○—○). At 180 min, an aliquot was placed at 30°C for 10 min and then returned (indicated by the arrow) to 40°C for the duration (●—●). 100 μg chloramphenicol/ml (CM) was added to an aliquot, and 5 min later, indicated by the arrow, the culture was transferred to 30°C (■—■). A portion of the culture was transferred to 30°C at 190 min (△—△). The amount of DNA was measured by ^{3}H-thymine incorporation as described in MATERIALS AND METHODS.

DNA synthesis occurred in the reverse experiment as well. After a culture of BW824 was starved for leucine at 30°C to terminate DNA replication, an aliquot was placed at 40°C and replenished with leucine 5 min later. DNA replication resumed, but at a rate between $1/5$ to $1/6$ of the 30°C control culture (Figure 5). *dna-325* mutants resemble the *dnaD* mutant strain PC-7 (CARL 1970) and the mutant strain F21 described by FANGMAN and NOVICK (1968) (and classified as a *dnaB* mutant by WECHSLER and GROSS 1971) in their ability to resume DNA synthesis at the restrictive temperature after a prior amino acid starvation at the permissive temperature. In contrast, DNA replication does not resume in a *dnaC* mutant strain PC-2 (CARL 1970) and *dnaA* mutants (KUEMPEL 1969; HIROTA, MORDOH and JACOB 1970; BEYERSMAN, SCHLICHT and SCHUSTER (1971).

Further evidence pointing to the reversible nature of the defect is seen in

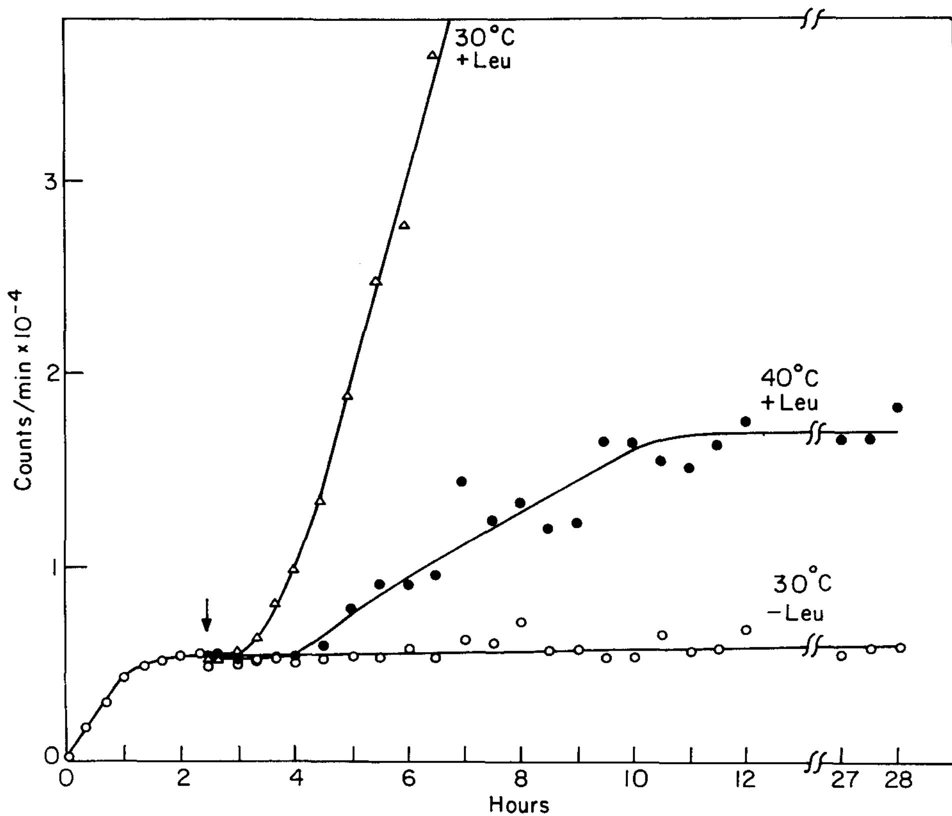

FIGURE 5.—DNA synthesis at 40°C following leucine starvation at 30°C. A culture of strain BW824 growing exponentially in minimal medium supplemented with leucine and thymine was transferred to medium without leucine. ^{3}H-thymine (12.5 μCi/ml) was added to 0 min. At 145 min an aliquot was removed to 40°C; 5 min later leucine was added to the 40°C culture as well as to a 30°C aliquot (indicated by arrow). ^{3}H-thymine incorporation into DNA was measured as described in MATERIALS AND METHODS. Leucine starvation for entire experiment (O—O); 40°C + leucine (●—●); 30°C + leucine (△—△).

Figure 4. Cooling the culture to 30°C for as little as 10 min allows further DNA replication (approximately 37% increase in the experiment shown; in another experiment a 20 or 30 min pulse at 30°C gave similar results to a 10 min pulse). The amount of DNA made is roughly half of that made in the presence of chloramphenicol at 30°C. That more DNA is replicated after a short 30°C pulse also can be seen by comparing Figures 2D and E. In Figure 2D the culture was maintained at 41°C for 240 min; in Figure 2E after 90 min at 41°C the culture was cooled to 30°C for 20 min and then replaced at 41°C for a total incubation time of 240 min. Hybrid DNA increased and heavy DNA appeared as well; the newly replicated DNA came to about 95% of the amount of DNA present at the beginning of the 41°C incubation. However, all of the light DNA did not disappear; whereas in control cultures grown at 30°C the light DNA was completely

converted to hybrid and heavy DNA in the same period of time. The amounts of DNA made in these two experiments and the pattern of DNA replication obtained in Figure 2E, particularly the persistence of light DNA, could be due to the occurrence of asymmetric initiation.

Chromosomal re-initiations are induced by temporarily blocking DNA synthesis by thymine starvation (PRITCHARD and LARK 1964), nalidixic acid (BOYLE, GOSS and COOK 1967), or heat in temperature sensitive DNA mutants (STEIN and HANAWALT 1969, WORCEL 1970). DNA synthesis in a *dnaB*⁻ strain stops immediately upon being placed at the restrictive temperature; a short pulse at the permissive temperature causes asymmetric re-initiation in only one of the two pairs present in a partially synthesized daughter chromosome (WORCEL 1970). Recovery from thymine starvation also induces asymmetric re-initiation (PRITCHARD and LARK 1964).

Because of this phenomenon, experiments similar to those carried out with *dnaA* mutants (HIROTA, MORDOH and JACOB 1970; ABE and TOMIZAWA 1971) were not done with *dna-325* mutants; in those experiments cells were pulse-labeled at 30°C after an incubation period at the restrictive temperature and then were returned to the restrictive temperature with a subsequent density shift at 30°C. The results showed that the pulse label was replicated early and the conclusion was that *dnaA* mutants terminated rounds of replication at the restrictive temperature and are therefore initiation mutants. Immediate stop temperature sensitive DNA mutants give essentially the same results due to premature initiation (STEIN and HANAWALT 1969, WORCEL 1970) thereby pointing out the ambiguity of this criterion which has been used for defining an initiation mutation.

Temperate phage growth at the restrictive temperature: Since all replicons are thought to carry unique information for their own DNA initiation (JACOB, BRENNER and CUZIN 1963) it was of interest to see if the temperate phage λ and P1 could grow in the temperature sensitive cells under conditions where host DNA replication had ceased. Phage λ can grow on strain BW824 at the restrictive temperature, even though infection occurred after the cells had been incubated at the restrictive temperature for 120 min. Figure 6A shows that at 41°C in the absence of phage very little ^{3}H-thymine is incorporated into DNA; whereas when the cells were infected with λ much more labeled DNA is found. (The cells were incubated at 41°C for 120 min before adding ^{3}H-thymine, so no label should have been incorporated into non-infected bacterial DNA. It is not known whether the small amount of radioactive label incorporated is due to DNA replication or repair.) In the particular experiment shown, a rather low burst size of 4 was obtained. An experiment was done which avoided the need to infect the cultures at 41°C. BW824(λ) and a lysogenic wild type strain DG10, as control, were induced with mitomycin after preincubation at 40°C for 120 min. Further incubation at the restrictive temperature for 150 min yielded burst sizes of 100 and 300 respectively.

Figure 6B shows the results observed when P1 was used as the infecting phage, under essentially the same experimental conditions that were used in the λ experiment. As in the case with λ, P1 infection causes DNA replication to occur

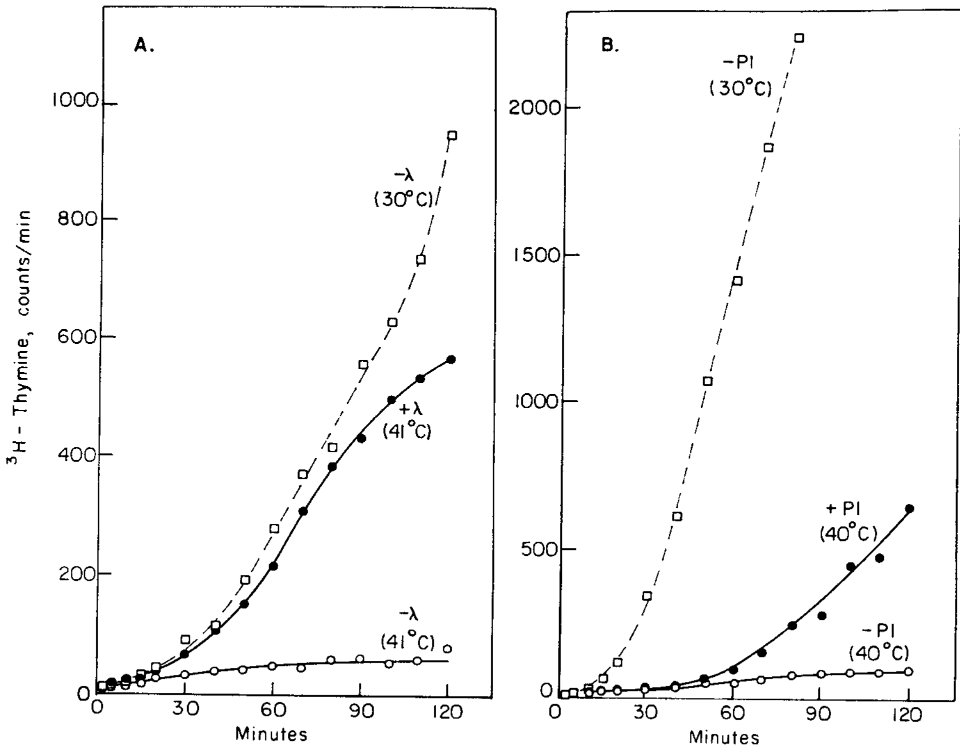

FIGURE 6.—Stimulation of DNA replication at the restrictive temperature by infection with λ or P1 phage. Cultures of BW824 were incubated at the restrictive temperature for 120 min prior to phage infection and ^{3}H-thymine addition. The detailed experimental procedure is described in MATERIALS AND METHODS. A. λ experiment. B. P1 experiment. Uninfected cells, incubated at the restrictive temperature (○—○); phage infected cells incubated at the restrictive temperature (●—●); uninfected cells incubated at 30°C (□—□).

at a temperature where cellular DNA synthesis is restricted. However, no appreciable amount of progeny phage was detected. The *dna-325* mutation may not be the reason why P1 progeny were not made at the restrictive temperature. Similar results were obtained in a *dnaA* mutant and its wild type parent with the conclusion that a host function responsible for P1 phage maturation and unrelated to the *dnaA* mutation was inactivated after an extended period at 42°C (BEYERSMANN and SCHUSTER 1971).

Nucleotide pools: Evidence that nucleotide pools in the mutant are comparable to those in the wild type strain is shown in Table 1. Essentially no decrease was found in any of the nucleotide triphosphates with increasing incubation times at the restrictive temperature. The temperature sensitive strain appears to contain somewhat more dATP and dGTP, but it is not known if these differences are significant. Anomalously high levels of dATP have been found in other DNA temperature sensitive mutants (FANGMAN and NOVICK 1968, CARL 1970) and in cells undergoing thymineless death (MUNCH-PETERSON and NEUHARD 1964).

TABLE 1

Nucleoside triphosphate pools at 41°C

Strain	Min*	ATP	CTP	GTP	UTP	dATP	dCTP	dGTP	TTP
	0	46.3	8.3	19.8	12.5	5.1	3.0	1.7	3.0
BW827†	40	43.9	11.2	18.2	8.8	10.5	2.0	2.0	3.2
	80	42.0	11.4	18.0	9.6	11.5	1.8	2.5	3.0
	120	41.6	11.5	16.6	10.6	11.8	2.0	2.9	2.8
	40	45.9	10.2	18.7	10.8	8.1	2.5	1.2	2.4
DG360	80	48.0	9.8	18.8	13.0	4.7	2.4	1.1	2.0
	120	48.9	7.4	21.2	12.1	5.5	2.0	1.0	1.6

Expressed in percent of total ^{32}P-labelled nucleotides recovered.
* Minutes after transfer to 41°C.
† In a parallel culture of BW827, DNA synthesis stopped after 50 min at 41°C.

The normal burst size of λ after mitomycin induction at 41°C as well as the results seen in the toluenized cell system, where the deoxyribonucleotide triphosphates are provided in the assay, also show that the temperature sensitive aberration is not due to a depletion of any of the nucleotide pools.

DNA replication in toluene-treated cells: DNA replication in toluene treated cells appears to reflect the replication that occurs *in vivo*. A *dnaB* mutant which is thought to be involved in chain elongation, stops DNA replication immediately when assayed in toluenized cells at the restrictive temperature. Furthermore a *dnaA* mutant continues making DNA in toluenized cells at the restrictive temperature (Mordoh, Hirota and Jacob 1970, Kohiyama and Kolber 1970) as would be expected for a mutant involved only in DNA initiation and not in replication. Since only a small fraction of the chromosome is replicated in the toluenized-cell assays, termination and re-initiation of the chromosome should not be involved. Typical results of DNA replication assays in toluene treated cells carrying *dna-325* are shown in Figures 7 and 8, and the results of these and other similar experiments are summarized in Table 2. As in the *in vivo* experiments, DNA replication can proceed in toluene treated cells at the restrictive temperature. In strain BW824 (as typified in Figures 7 and 8 A,B), the amount of DNA made at 40°C averaged about 60% of that made at 30°C. (The range observed in various experiments was between 44 and 86%). On the other hand, if mutant cells were preincubated for 180 min at 40°C and then treated with toluene and assayed at 40°C, the amount of DNA replication decreased to as little as 8% of the control value (Figure 7D). This value can be compared to the amount of ^{3}H-dCTP incorporation found in toluene treated cells which were previously starved for a required amino acid in order to terminate the rounds of chromosomal replication (Table 2). Cells pretreated at 40°C but then assayed at 30°C made about 15% DNA as compared to the control (Figure 7C), indicating that in the toluene system a certain amount of reversibility may occur, as *in vivo*.

Of interest is the observation that in the toluene assay the wild-type strain DG75 is itself somewhat temperature sensitive if the culture is first pre-incubated

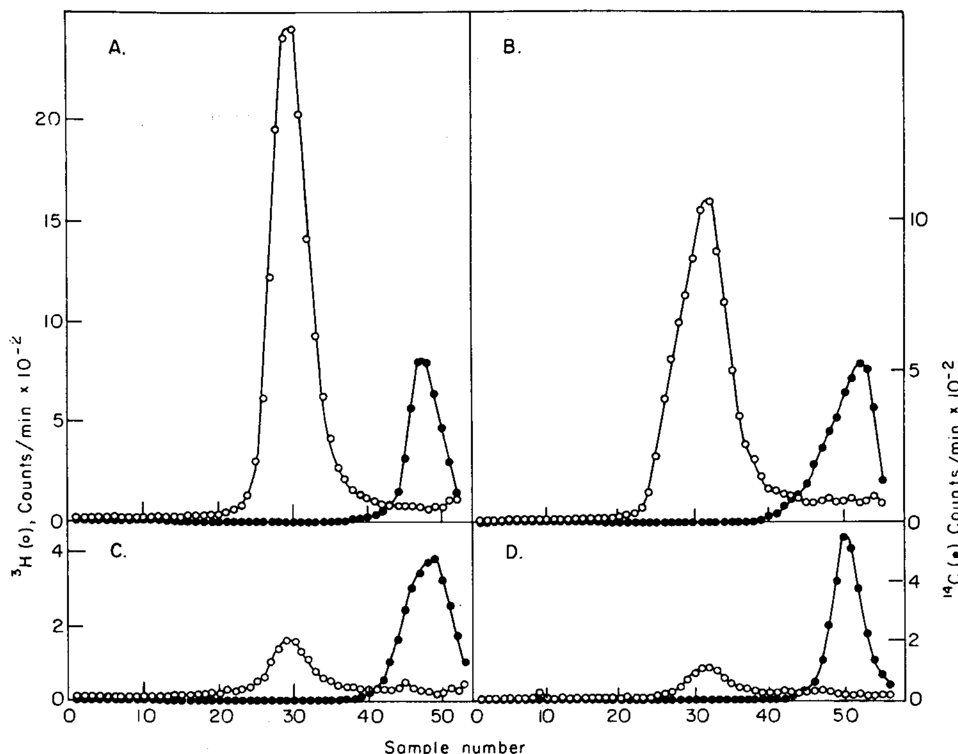

FIGURE 7.—Density gradient analyses of DNA made in toluene treated cells of strain BW824. Cells were grown over six generations in the presence of ^{14}C-thymine, 0.055 μCi/ml (●—●). The reaction mixture after toluene treatment contained deoxybromouridine triphosphate and ^{3}H-dCTP (O—O). Half of the culture was grown at 30°C, the cells harvested, treated with toluene, reaction mixture added and assayed at A. 30°C, B. 40°C. The remainder of the cells were incubated at 40°C for 180 min, harvested at 40°C, treated with toluene, reaction mixture added and assayed at C. 30°C, D. 40°C. The detailed procedure is described in MATERIALS AND METHODS. The majority of the ^{3}H-label was found at a density expected for hybrid DNA.

at 40°C for 180 min (Table 2). Although DG75 grows normally at 40°C, some cell death is observed at 41°C, and as noted above (MATERIALS AND METHODS), the *in vivo* DNA replication rate is somewhat lower at 41°C than at 40°C in strain DG75. This apparent temperature sensitivity in the wild-type strain has not been investigated.

It should be noted that even at the permissive temperature the amount of replication was roughly 7-fold lower in the temperature sensitive strain BW824 as compared to its wild-type parent DG75 (Table 2). The wild-type strain incorporated about 1.5×10^5 dCTP molecules per cell, which would be equivalent to about 9% of the chromosome, assuming 3.2×10^6 base pairs per chromosome (STENT 1971). (Roughly half this value was obtained when the percent of ^{14}C label in the hybrid region was determined rather than using the amount of ^{3}H label incorporated into the hybrid DNA.) On the other hand, the strain carrying

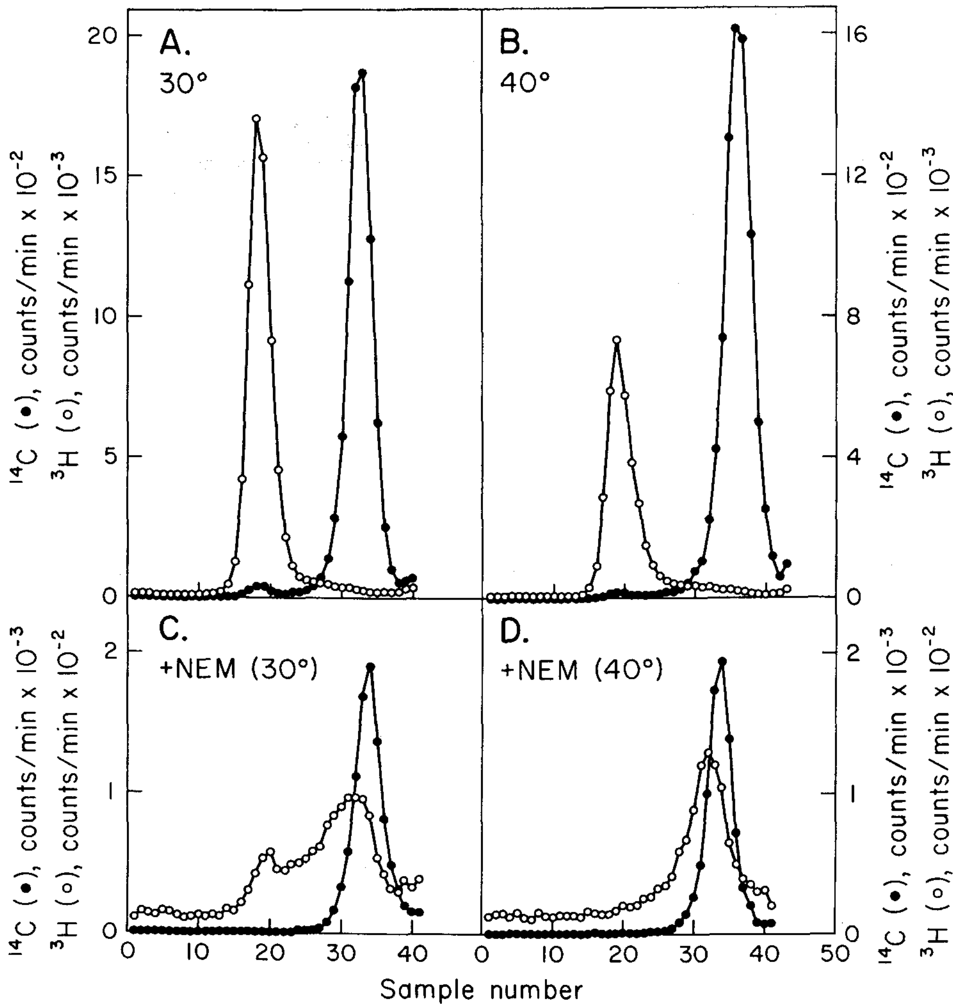

FIGURE 8.—The effect of N-ethylmaleimide on DNA made in toluene treated cells of strain BW824. Cells were grown at 30°C for seven generations in the presence of ^{14}C-thymine, 0.055 μCi/ml (●—●). The reaction mixture after toluene treatment contained deoxybromouridine triphosphate and ^{3}H-dCTP (O—O). The cells were harvested, treated with toluene, reaction mixture added and assayed at A. 30°C, B. 40°C, C. 30°C in the presence of 1.2 mM N-ethylmaleimide (NEM), D. 40°C in the presence of 1.2 mM N-ethylmaleimide. Note the change in ordinate scales between the top and bottom curves, particularly the >10× magnification in the ^{3}H scales in C and D as compared to A and B.

dna-325 incorporated about 2×10^4 dCTP molecules per cell, equivalent to replication of about 1.2% of the chromosome. Consequently in cells pretreated at 40°C and then also assayed at the restrictive temperature, as few as 1.5×10^3 dCTP molecules were incorporated, equivalent to or less than 0.1% of the chromosome. Under the same 40°C pre-incubation conditions, about 5% chromosomal replication occurred in strain DG75.

TABLE 2

DNA replication in toluenized cells

Strain	Culture conditions	Assay temperature (°C)	Percent uptake of ^{3}H-dCTP relative to 30° exponential*	dCTP molecules incorporated/cell	Number of experiments averaged
BW824	30°, exponential	30°	100	2×10^4‡	7
	30°, exponential	40°	60		7
	— leucine	30°	4.7		3
	— leucine	40°	1.6		2
	40°, 180 min	30°	15		4
	40°, 180 min†	40°	8.5		5
DG75	30°, exponential	30°	100	1.5×10^5‡	3
	30°, exponential	40°	94		3
	40°, 180 min	30°	56		1
	40°, 180 min	40°	53		1

A summary of experiments of the types seen in Figures 7 and 8. The experimental procedures are described in MATERIALS AND METHODS.

* The areas under the ^{3}H curves (e.g., Figure 7) were first normalized to the amount of DNA present in each gradient as measured by the total amount of light-banding ^{14}C overnight labeled DNA, then the 30°C control was made equal to 1 and the other gradients were compared to this value.

† In one experiment the culture was incubated at 40°C for 240 min with the same results as the 180 min incubation.

‡ The values were obtained by knowing the specific activity of the ^{3}H-dCTP, the number of cells used, and the amount of ^{3}H recovered in the hybrid DNA peak. The values are approximate as they do not take into consideration possible cell or DNA losses during the experiment.

Figure 8C and D show that the amount of replication of hybrid DNA in the presence of N-ethylmaleimide is insignificant, even though the cells that were used are *polA*$^+$. Pol I is not inhibited by N-ethylmaleimide in the toluene-treated cells assay (MOSES and RICHARDSON 1970). The small amount of ^{3}H-label present in the N-ethylmaleimide-treated cultures which bands predominantly in the lighter regions is most likely due to repair synthesis (HANAWALT *et al.* 1968).

In vitro enzyme assays: The above results are based on toluene treated cells. No differences could be detected in the heat lability of polymerase I or II activities purified from BW824 when compared with the same activities purified from DG75 (C. C. RICHARDSON, personal communication).

Effect of deoxycholate on cells: A *dnaA* mutant is significantly lysed in the presence of deoxycholate at the restrictive temperature and the mutation is believed to involve the bacterial membrane (HIROTA, MORDOH and JACOB 1970). By this criterion at least, the *dna-325* mutation does not appear to affect the cell membrane; 0.5% deoxycholate caused a moderate decrease in optical density in a mutant culture (BW827) at 40°C but to no greater extent than the wild type (AT2459) control (data not shown).

Genetic mapping of dna-325: Interrupted matings indicated that *dna-325* mapped about 2.5 to 3 min away from *leu*. Further testing showed that *dna-325* cotransduced with *serB*. In cross 1 (Table 3) the cotransduction frequency between *dna-325* and *serB* was found to be 52% while that between *dra* and *serB*

TABLE 3

Transduction frequencies

Cross	Donor	Recipient	Selected transductants	Unselected markers	Dra⁻	Dra⁺	Total
1.	BW824 (*dna-325, dra⁻*)	AT2459 (*serB22*)	Ser+	T^s	193	12	386
				T^r	98	83	
2.	AT2459 (*serB22*)	BW825 (*dna-325, dra⁻*)	T^r	Ser+	126	104	309
				Ser−	0	79	
3.	WA993 (*hsp⁻*)	BW825 (*dna-325, dra⁻*)	T^r	Hsp−	38	33	224
				Hsp+	53	100	

T^s = Temperature-sensitivity, T^r = Temperature-resistance.
P1 phage were grown on the donor strains and were used to transduce the recipient strains.

was 74%. This indicated that *dra* mapped closer to *serB* than did *dna-325*. The reciprocal cross (number 2 in Table 3) gave a lower cotransduction frequency between *dna-325* and *serB* (26%), but showed a cotransduction frequency of 59% between *dra* and *dna-325*; these data are thus consistent with those shown in cross 1 with regard to the relative positions of these three markers. Furthermore, cross number 3, with cotransduction frequencies of 59% between *dna-325* and *dra* and 33% between *dna-325* and *hsp*, places *dna-325* between *dra* and *hsp*. Cross 4 (Table 4) gave similar results for the cotransduction frequency of *hsp* and *dna-325*. Cross 5 (Table 4) yielded a co-transduction frequency between *hsp* and *serB* of 16%, and crosses 6 and 7 (Table 4) gave co-transduction values of 7–12% between *serB* and *thr*. The map positions of the various markers used, consistent with the data in Tables 3 and 4 are summarized in Figure 9.

Spontaneous temperature resistant "revertants": The reversion rate to temperature resistance is about 2×10^{-6}. Five colonies of BW828 which grew up on NYA plates at 41 °C were examined; all five still contained the temperature sensitive mutation. This was shown by infecting AT2459 with P1 which was grown

TABLE 4

Transduction frequencies

Cross	Donor	Recipient	Selected transductants	Unselected marker		Total
				Hsp⁺	Hsp⁻	
4.	WA993 (*hsp⁻*)	BW827 (*dna-325*)	T^r	94	50	144
5.	WA993 (*hsp⁻*)	AT2459 (*serB22*)	Ser+	115	22	137
				Thr⁺	Thr⁻	
6.	AB1157 (*thr⁻*)	AT2459 (*serB22*)	Ser+	42	6	48
7.	AB1157 (*thr⁻*)	AT2459 (*serB22*)	Ser+	174	13	187

T^r = Temperature resistance.

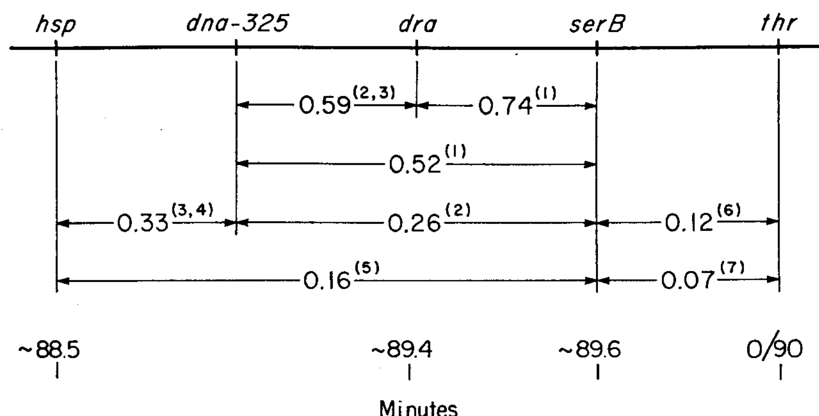

FIGURE 9.—The map position of *dna-325* on the *E. coli* chromosome. The numbers shown are the co-transduction frequencies obtained for the various markers. The numbers in parentheses refer to the crosses shown in Tables 3 and 4. The relative time positions of the other markers are according to TAYLOR (1970).

on the "revertants", selecting for Ser+ colonies at 30°C and examining these purified colonies for their response to growth at 30°C and 41°C. The results are shown in Table 5. In all five cases temperature sensitive colonies were found. Due to the appearance of colonies with questionable growth at 41°C, exact transduction frequencies could not be determined, but the results are consistent with a transduction frequency of about 50% between *dna-325* and *serB* as is shown in Figure 9. As comparison for the spontaneous temperature-resistant "revertants", a temperature resistant strain (BW832) was prepared by transduction and then used as the donor; no temperature sensitive colonies were found among the Ser+ transductants. Since the spontaneous temperature resistant "revertants" still contain the temperature sensitive trait, their phenotypic behavior is probably

TABLE 5

Existence of the temperature sensitive trait in phenotypically temperature resistant strains

Donor	Colony number	Unselected marker T^s	Partially T^{r*}	T^r
BW831	1	20	8	20
	2	14	4	29
	3	9	17	21
	4	20	13	14
	5	6	6	35
BW832	1	0	0	48
	2	0	0	47

P1 phage were grown on the donors shown; all are phenotypically temperature resistant. The recipient in all transductions was AT2459; the selected marker was Ser+.
* Slight growth at 41°C, which upon retesting continued to give equivocal results.
T^s = Temperature sensitive; T^r = Temperature resistant.

due to suppression. However none of the strains was able to sustain the growth of T4 amber, ochre, or umber mutants. The location of the suppressor has not been mapped, and it is not known what type of suppression is occurring. Further investigation of these "revertants" may prove to be interesting since they may have increased levels of a compensating enzyme activity.

Recessive nature of dna-325: When the wild-type allele of *dna-325* carried by the episome KLF1 was introduced into strain BW833, the cells became phenotypically temperature resistant. Since the recipient strain was *recA*⁻, it was considered most unlikely that the temperature resistance was due to integration of the wild-type allele into the chromosome replacing the mutated *dna-325*. It was impossible to confirm the presence of the F′ by acridine orange curing (BASTARRACHEA and WILLETTS 1968), since the presence of *recA*⁻ made the cells extremely sensitive to acridine orange. However through transduction experiments it was possible to demonstrate that P1 grown on the phenotypically temperature resistant cells were still able to cotransduce temperature sensitivity with Ser^+ into AT2459. Hence the phenotypically temperature resistant cells still contained the temperature sensitive mutation. Therefore *dna-325* is recessive to its wild-type allele, and the function is *trans* acting.

DISCUSSION

Proof of the existence of DNA initiation mutants necessarily has depended upon a summation of results from many different types of experiments since, as yet, no direct assay for DNA initiation can be performed. Although some of the criteria used for defining initiation mutants are ambiguous, the results suggest that DNA initiation is blocked in *dna-325* mutants, but the block is a leaky one and some initiation can occur at the restrictive temperature.

Data analysis: (1) the fact that more than two cell divisions occurred at the restrictive temperature (Figure 1) would indicate that at this temperature most chromosomes had terminated. Cell division appears to be triggered by termination of chromosomal replication (HELMSTETTER and PIERUCCI 1968, CLARK 1968). Furthermore after a return to the permissive temperature, cell division occurred only after the content of DNA in the cells had doubled. If the mutation did not affect initiation but caused replication to cease in all chromosomes after approximately 50% more replication, it would be expected that cell division would begin very shortly after the culture was replaced at the permissive temperature.

(2) If termination of all chromosomes occurred during the 40°C incubation, a shift to 30°C in the presence of chloramphenicol would result in a 100% increase in DNA over the amount present at the time of the shift, if all chromosomes re-initiate and again replicate to completion. If, on the other hand, the mutation in *dna-325* caused synthesis to stop after some chain elongation on *all* chromosomes, including initiation on those which terminated, this population would resemble an exponential population, and the addition of chloramphenicol upon return to the permissive temperature should yield the expected 40%

increase in DNA. A DNA increase of 80% was found in strain BW824 (Figure 4). A similar DNA increase of 80% was observed in a *dnaA* mutant (BEYERSMANN, SCHLICHT and SCHUSTER 1971). When the chloramphenicol was added 20 min prior to the shift to 30°C, about 50% more DNA was made indicating that not enough initiator protein had accumulated to result in initiation in all of the population. The 80% increase suggests that rounds were completed during the 40°C incubation but that subsequently at 30°C some chromosomes did not initiate new rounds of replication.

(3) Consistent with the hypothesis that *dna-325* may be involved in initiation is the observation that synthesis of λ (and perhaps P1) DNA occurs under conditions where host DNA replication has stopped (Figure 6). The ability for λ to grow at the restrictive temperature has been used as a criterion for the existence of an initiation mutant (HIROTA, MORDOH and JACOB 1970; CARL 1970; BEYERSMANN, SCHLICHT and SCHUSTER 1971; GROSS 1972). The argument is based on the Replicon Model (JACOB, BRENNER and CUZIN 1963) where a mutation affecting host initiation should not affect the initiation of DNA replication in independent replicons such as λ. However the mere fact that λ progeny can be made at the restrictive temperature does not preclude the possibility that the phage provide or do not require the *dna-325* function, a function which may have nothing to do with initiation.

(4) Residual DNA synthesis occurs after the shift to 40°C in amounts consistent with those expected for an initiation mutant; the results with the toluenized cells (Figures 7 and 8; Table 2) can also be explained by a defect in DNA initiation. It should be noted that these results are consistent with but do not prove the proposed defect in DNA initiation. Similar results would be obtained from a chain elongation defect where DNA replication persists for a time at the restrictive temperature before stopping.

(5) The resumption of DNA synthesis at 40°C after prior amino acid starvation at 30°C (Figure 5) and the existence of heavy DNA made at 40°C in the presence of BU (Figures 2B, C and 3) both suggest that if the mutation does inactivate initiation of replication, this inactivation is incomplete, i.e., the mutation is "leaky" and some further initiation events can occur at the restrictive temperature. The results are ambiguous however since they can also be explained by a chain elongation defect.

(6) A short incubation period at 30°C in the midst of a long 40°C incubation period led to doubling of the DNA content in a *dnaA* mutant (HIROTA, MORDOH and JACOB 1970). However only 37% more DNA was made in strain BW824, roughly half of that which was made in the presence of chloramphenicol at 30°C after a 40°C incubation (Figure 4). A discrepancy exists in the amount of DNA made under these two conditions. The amount of DNA made in the presence of chloramphenicol at 30°C after a 40°C incubation is consistent with an initiation mutation, as discussed above. The amount of DNA made after a short 30°C pulse cannot be explained at this time; the phenomenon of asymmetric re-initiation of DNA synthesis caused by inhibition of DNA synthesis may be involved (PRITCHARD and LARK 1964, WORCEL 1970).

Genetic classification: The locus *dna-325* was classified as a *dnaC* mutation by GROSS (1972) based on information which I gave him concerning the map location of *dna-325* and the ability of *dna-325* mutants to support the growth of λ at the restrictive temperature. *dnaC* mutants have been classified as initiation mutants (CARL 1970, WECHSLER and GROSS 1971, SCHUBACH, WHITMER and DAVERN, in press) and are cotransducible with *dra* at a 20–50% frequency. Also mapping near *dra*, with a 50% cotransduction frequency, was the locus *dnaD* (strain PC-7, CARL 1970); this locus was distinguished from the *dnaC* locus (strain PC-2) because of the mutant's inability to grow λ at the restrictive temperature. Recently however WECHSLER (1973) has shown that *dnaC* and *dnaD* both define a single complementation group, and he has suggested that the *dnaD* notation be deleted. Since *dna-325* mutants have characteristics of both strain PC-2 and PC-7 it is most likely that *dna-325* is in locus *dnaC*, but complementation tests must be done to confirm this.

The results of experiments to measure whether *dna-325* mutants can undergo integrative suppression (NISHIMURA *et al.* 1971) have thus far been indeterminate.

Conclusions: Many of the results are consistent with the view that the *dna-325* mutation is one involved with DNA initiation. However, the fact that heavy DNA is made when *dna-325* mutants are incubated at 40°C in the presence of BU suggests the possibility that some initiation can occur at that temperature. Therefore if *dna-325* mutants are blocked in DNA initiation, the block must be a leaky one.

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