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Conditional Change of DNA Replication Control in an RNA Polymerase Mutant of *Escherichia coli*

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A temperature-sensitive mutant of *Escherichia coli* with a temperature-dependent change in the control of initiation of DNA replication was isolated. The phenotype of the mutant was dependent on a mutation in the RNA polymerase gene *rpoC*. In vitro RNA polymerase activity was temperature sensitive. The mutant grew and synthesized DNA at 30°C as did the wild type. After a shift to 39°C, a temperature still permissive for growth, the mutant increased its origin concentration more than twofold. After a shift from 39 to 30°C, initiation of DNA replication was inhibited until the normal origin concentration was reestablished.

The duplication of chromosomes in *Escherichia coli* is regulated at the level of initiation. Growing cells initiate new rounds of DNA replication when they reach a certain cell mass per chromosomal origin, the initiation mass, and the average origin/mass ratio varies little with growth rate (9, 21, 23).

A temperature-sensitive *dnaA* mutant has been used to analyze the regulatory interplay between mass synthesis and initiation of DNA replication. A shift to a temperature intermediate between permissive and nonpermissive causes a reduction in the origin/mass ratio, probably due to a less active *dnaA* protein that becomes limiting for the initiation of DNA replication (12). The *dnaA* protein interacts with the RNA polymerase (1, 24) in the transcriptional step required for the initiation of DNA replication (14).

Here we describe a temperature-sensitive mutant in which the origin/mass ratio increases twofold at a semipermissive temperature. Genetic mapping and biochemical analysis showed that this phenotype is dependent on a mutation in the *rpoC* gene that codes for the β′ subunit of the RNA polymerase.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains used are listed in Table 1. The cultures were grown in AB medium (6) supplemented with 0.2% glucose plus one or more of the following when required: thiamine, 2 μg/ml; adenine sulfate, 60 μg/ml; thymine, 10 μg/ml. Amino acid requirements were met by the addition of 0.5% Casamino Acids or a mixture containing all amino acids except methionine and cysteine to a final concentration of 20 μg/ml for the aromatic amino acids, 100 μg/ml for leucine, and 50 μg/ml for the others.

Cell mass was determined as optical density at 450 nm (OD_{450}) in a Zeiss PMQ 2 spectrophotometer.

DNA was determined by the diphenylamine method (5) or by the incorporation of [2-^{14}C]thymine into trichloroacetic acid (TCA)-precipitable material (12).

Origins were estimated by the principle used by Hansen and Rasmussen (12); in this case, culture samples were treated with chloramphenicol (200 μg/ml) to stop initiation and incubated for 3 h, which was found to be adequate for DNA synthesis to be completed.

Mutant isolation procedure. A culture of strain CP385 was treated as follows. The cells were mutagenized with nitrosoguanidine and grown at 30°C, then shifted to 39°C for 45 min (to allow for excessive initiation in the desired mutant cells). The culture was then starved for arginine for 90 min at 30°C (to stop initiation and to allow ongoing rounds of replication to terminate) and then grown in medium without thymine but with arginine for one mass doubling at 30°C (to prepare nonmutant cells for initiation. The desired mutant cells should, due to their high origin/mass ratio, require longer growth before being ready for initiation). The culture was then grown in 5-bromouracil plus arginine at 30°C for 20 min (to let nonmutant cells incorporate 5-bromouracil into their DNA and thus photosensitize them to UV light [4]) and irradiated with long-wave-length UV light (to kill nonmutant cells preferentially). All steps after mutagenesis were repeated once. Temperature-sensitive (42°C) mutants were screened among the survivors, and screening for strains with a high DNA/mass ratio was done at 39°C.

Assay of RNA polymerase activity. RNA polymerase activity was measured in the following mixture, which in 1 ml contained 25 mM Tris-hydrochloride (pH 7.9), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 50 μg of bovine serum albumin, 10 μg of λd*ilv5* DNA (13),

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0.1 M KCl, 0.2 mM of CTP, GTP, and ATP, and 0.05 mM UTP, 10 μCi of [^{3}H]UTP, and 50 μl of RNA polymerase extract (see below). The reaction was started after 5 min of preincubation by the addition of the nucleoside triphosphates. This was done, with minor modifications, as described by Cross et al. (8). Samples (100 μl) were taken at intervals into 2 ml of TCA–20 μg of yeast RNA per ml, and the incorporation into TCA-precipitable material was determined.

The extracts assayed for RNA polymerase activity were prepared as fraction iv, as described by Cross et al. (8), dialyzed against 50% glycerol–10 mM Tris-hydrochloride (pH 7.9)–5% polyethylene glycol–0.1 M KCl–1mM dithiothreitol and stored at −20°C.

Genetic mapping. Hfr mapping was done as described by Low (17) and P1 transduction as described by Lennox (16).

Complementation analysis. The ColE1-derived plasmids (7) were introduced into the recipient cells by mobilization with F'lac (from strain NF1010), selection for the Rif^r allele carried by the plasmids, and screening for colicin E1 immunity. The pBR322-derived plasmids (obtained from N. Fiil [10]) were introduced by transformation (18) and selection for ampicillin-resistant colonies.

RESULTS

The mutant described in the present study (strain CP387) was isolated by the procedure outlined above. The parent and mutant strains grew at nearly the same rate and had nearly the same DNA/mass ratio at 30°C. Balanced growth of the mutant could be obtained at 39°C, but its growth rate was reduced relative to the parent, and cell size as well as the DNA/mass ratio were increased more than twofold. The mutant did not form colonies at 42°C.

Genetic analysis. Cross-streaking tests with several Hfr strains indicated that the temperature-sensitive mutation(s) in strain CP387 was located within the region of the chromosome defined by the points of entry of the HfrRa-2 (87 min) and Hfr209 (90 min) (17). To map the mutations, we used two selective markers located in this region, *argE,H* and *purD*, in the P1 transductions (Table 2). Three mutations were identified, two conferring temperature-sensitive growth, Ts-1 and Ts-2 (Table 2, experiments A and B), and one, Das (*dnaA* suppression), suppressing the temperature sensitivity of strains carrying the *dnaA46* allele (Table 2, experiment C).

The transduction in experiment A revealed the presence of a mutation, Ts-2, located between *argH* and *rpoB*(Rif). The transductants carrying this mutation grew normally at 30 and 39°C, but were temperature sensitive for growth at 42°C. Another mutation, Ts-1, conferring a

TABLE 1. *E. coli* strains

Strain(derivation)	Sex	Genotype of the chromosome[a]	Source, reference, or construction
CP385 (K-12)	Hfr	*thi argA thyA deoB supE42*	Derivative of W1485
CP387 (K-12)	F$^-$	*thi argA thyA deoB supE42* Ts-2 *rpoB906 rpoC907*	This work
Ra-2 (K-12)	HfrRa-2	*mal-28 supE42*	17
KL209 (K-12)	Hfr209	*malB16 sup-53*	17
MA1079 (K-12)	HfrKL16	*thi serA recA*	J. Davies via N. Fiil
CM139 (AS19)	F$^-$	*leu argH* Rif	K. von Meyenburg
AB468 (K-12)	F$^-$	*thi-1 proA2 mtl-1 xyl-5 galK2 lacY1 supE44*? *his-4 purD13*	B. Bachmann
FH72 (K-12)	F$^-$	As for AB468, plus Rif	Spontaneous Rif of AB468
FH56 (K-12)	F$^-$	*thi-1 proA2 mtl-1 xyl-5 galK2 lacY1 supE44*? *his-4* Rif Ts-2	This work (Table 2, expt B)
FH57 (K-12)	F$^-$	*thi-1 proA2 mtl-1 xyl-1 galK2 lacY1 supE44*? *his-4 rpoB906 rpoC907*	This work (Table 2, expt B)
TC496 (K-12)	F$^-$	*thi-1 proA2 mtl-1 xyl-5 galK2 lacY1 supE44*? *rpoB906 rpoC907 recA*	MA1079 × FH57, select His$^+$
JF923 (K-12)	F$^-$	*thi his leu*(Am) *cdd lac*(Am) *supD*(Ts) Nal *ilv argE* Rif	J. Friesen
TC204 (K-12)	F$^-$	*thi his leu*(Am) *cdd lac*(Am) *supD*(Ts) Nal *dnaA46 argE* Rif	P1(CM734) (11) × JF923, select Ilv$^+$
TC210 (K-12)	F$^-$	*thi his leu*(Am) *cdd lac*(Am) *supD*(Ts) Nal *dnaA46 rpoB906*	This work (Table 2, expt C)
WM301 (B/r)	F$^-$	*leu proA trp his arg thyA deoB lac gal hsd^{K-12} rpsL metB*	W. Messer (3)
TC92 (B/r)	F$^-$	*leu proA trp his arg thyA deoB lac gal hsd^{K-12} rpsL*	P1(FH57) × WM301, select Met$^+$
TC98 (B/r)	F$^-$	*leu proA trp his arg thyA deoB lac gal hsd^{K-12} rpsL rpoB906 rpoC907*	P1(FH57) × WM301, select Met$^+$

[a] For genetic symbols, see reference 2.

TABLE 2. P1 transduction analysis of mutations in strain CP387

Expt	Donor strain	Recipient strain	Selected marker	Unselected markers[a]			No. of transductants
				Ts-2 (A, B) or Das (C)	Rif	Ts-1	
A1	CP387 (*argH*$^+$ Ts-2 Rif^s Ts-1)	CM139 (*argH* Rif$^+$)	*argH*$^+$	R	R	R	16
2				D	R	R	4
3				D	D	R	0
4				D/R	D	R	94
B1	CP387 (*purD*$^+$ Ts-2 Rif^s Ts-1)	FH72 (*purD* Rif^r)	*purD*$^+$	R	R	R	60
2				R	R	D	2
3				R	D	D	12
4				D	D	D	148
5				D	R	R	2
C1	FH57 (*argE*$^+$ Das Rif^s Ts-1)	TC204 (*argE* Rif^r *dnaA46*)	*argE*$^+$	R	R	R	97
2				D	R	R	10
3				D	D	R	5
4				D/R	D	D	37
5				D/R	R	D	1

[a] Scoring for unselected markers was done as follows. (Experiment A) Ts-2: no colonies at 42°C, normal growth at 39 and 30°C; Ts-1: no colonies at 42°C, reduced growth rate at 30 and in particular at 39°C. (Experiment B) Ts-2: temperature sensitive for growth at 42°C on minimal medium only, normal growth at 39°C. Ts-1: no colonies at 42°C (on rich or minimal medium), reduced growth rate at 39°C. Temperature-sensitive transductants were also screened by microscopic observation of clones incubated for 24 h at 42°C on minimal medium; filamentous cells were formed only by strains with the growth characteristics of Ts-1, some (B2 and B3) giving rise to 10 to 30 long filaments and others (B4) forming 1 to 2 short filaments. Other transductants (B5) formed 4 to 10 normally sized cells, and they had the growth characteristics of Ts-2. Some transductants (B1) grew normally at all temperatures. (Experiment C) Ts-1: no colonies at 39°C, very slow growth at 30°C. Das: suppression of the temperature-sensitive phenotype of the *dnaA46* mutation, i.e., colony formation at 42°C. For all experiments: D, allele derived from donor; R, allele derived from recipient; D/R, allele origin could not be determined.

reduced growth rate at 30 and especially at 39°C, was found to be located close to the *rpoB*(Rif) mutation of the recipient strain and was not separated from it in this experiment.

In experiment B, the mutation responsible for the Ts-1 phenotype was separated from the *rpoB*(Rif) site and shown to be located clockwise of it. It was also shown to confer temperature sensitivity by itself (B2 and 3). Temperature-sensitive *purD*$^+$ transductants were screened for growth characteristics and cell morphology after incubation at 42°C. Three different types could be distinguished in this way. Only those classes giving rise to filaments at 42°C showed the reduced growth rate at 39°C attributed to Ts-1 in experiment A. Two other transductants (B5) were probably caused by a double crossover, which uncovered the Ts-2 mutation located between *argH* and *rpoB*(Rif) according to experiment A.

One of the transductants (B5) carrying only the Ts-2 mutation (strain FH56) and one of the Rif^s transductants (B3) carrying only the Ts-1 mutation (strain FH57) were chosen for further studies, including complementation analysis (Table 3; see below), which confirmed that these two strains carried different temperature-sensitive mutations. Strain FH57 (Table 4) but not strain FH56 (data not shown) showed the increased DNA/mass ratio at 39°C characteristic of the original mutant (see Fig. 3A), indicating that this phenotype is associated with the Ts-1 mutation. Spontaneous temperature-resistant revertants of strain FH57 had simultaneously reverted the slow growth and filament formation at high temperature.

Strain FH57 was used as donor in the P1 transduction for experiment C (Table 2). The recipient strain carried the *dnaA46* mutation, which affects the initiation of DNA replication at high temperature. This experiment was carried out to test whether a mutation causing an increase in DNA concentration at high temperature could suppress a mutation causing a decreased DNA concentration (12). The experiment indicated that strain FH57 carried a mutation (Das) conferring suppression of the temperature sensitivity of the *dnaA46* mutation, i.e., giving rise to temperature-resistant transductants. The transduction showed that the Das mutation was closely linked to and located counterclockwise of the *rpoB*(Rif) allele of the recipient strain. Control transduction showed that the Das mutation was not present in strain AB468, the parent of strain FH57 (data not shown). This indicates that it most likely comes from the

TABLE 3. Complementation analysis of the mutations in the chromosomal *rpoB, C* region of strain CP387

Plasmid[a]	Intact genes carried by plasmid	Complementation of mutations:[b]		
		Ts-2[c]	Das[d]	Ts-1[e]
pJC701	*rplA rplJ rplL rpoB*	NT	+	−
pJC702	*rplA rplJ rplL rpoB*	NT	+	−
pJC703	*rplA rplJ rplL rpoB rpoC*	NT	+	+
pJC720	*rpoB rpoC*	NT	+	+
pNF1492	*rplJ rplL rpoB*	−	NT	−
pNF1310	*rplL rpoB rpoC*	−	NT	+

[a] See Fig. 1 for structure of the plasmids.
[b] NT, Not tested; +, complementation; −, no complementation.
[c] The plasmids were introduced into strain FH56, and complementation of the Ts-2 mutation was tested by plating on minimal medium at 42°C.
[d] The plasmids were introduced into strain TC210, which carries both the *dnaA46* and Das mutations. Complementation was scored as loss of the ability to form colonies at 42°C.
[e] The pJC plasmids were introduced into strain FH57 and the pNF plasmids into strain TC496 (a *recA* derivative of strain FH57). Complementation was tested as the ability to form colonies at 42°C.

original mutant strain, CP387. The transductants that also received the Ts-1 mutation became even more temperature sensitive than the recipient strain, i.e., they did not grow at 39°C. Their very slow growth at 30°C is probably the reason for the unusually low cotransduction frequency between *argE* and Rif^r observed in this experiment.

These P1 transductions showed that the original mutant strain CP387 carried three mutations in the *rpoB,C* region of the chromosome and indicated the following gene (allele) order: *argE,H*–Ts-2–Das–*rpoB*(Rif)–Ts-1–*purD*.

The complementation experiments (Table 3) were carried out to map these mutations more precisely. Six different plasmids carrying various segments of the *rpoB,C* region were introduced into strains carrying the different mutations. The extent of chromosomal DNA carried by these plasmids is shown in Fig. 1. *rpoB* is the only intact gene common to the six plasmids. Three of the plasmids carry in addition the intact *rpoC* gene (pJC703, pJC720, and pNF1310). The temperature-sensitive mutation Ts-2, present in strain FH56, was not complemented by plasmids pNF1310 or pNF1492, nor was recombination to temperature resistance observed (Table 3). This indicates that the Ts-2 mutation is located outside the *rpoB,C* genes. The Das mutation, suppressing the temperature sensitivity of the *dnaA46* mutation, was complemented by the four pJC plasmids and must therefore be an *rpoB* allele. It was designated *rpoB906*. The temperature sensitivity of strains carrying the Ts-1 mutation was only complemented by the three plasmids that carried an intact *rpoC* gene. pNF1310, which contains the least chromosomal DNA after the *rpoC* gene (Fig. 1), carries an open coding sequence of 120 amino acid equivalents to the right of the *rpoC* gene. This coding sequence is, however, not normally expressed in vivo (25). The most likely position for the Ts-1 mutation is therefore inside the *rpoC* gene. The mutation was designated *rpoC907*.

In vitro analysis of RNA polymerase activity. Partially purified RNA polymerase was prepared from wild-type strain AB468 and mutant strain FH57, which carries the *rpoC907* and *rpoB906* alleles. At 30°C all of the partially purified RNA polymerase preparations showed linear RNA synthesis for more than 10 min (data not shown). The in vitro temperature shift experiments showed that after a shift to 40°C the RNA-synthesizing activity in the wild-type extract (Fig. 2A) increased twofold, whereas the activity in the mutant extract (Fig. 2B) was severely reduced. Partially purified RNA poly-

TABLE 4. Complementation analysis of the DNA replication phenotype of the *rpoB906*(Das) *rpoC907*(Ts-1) double mutant

Strain[a]	Plasmid[b]	Doubling time (min) at:			DNA[c] (μg/[ml · OD_{450}]) at:		
		30°C	39°C	Ratio (39/30°C)	30°C	39°C	Ratio (39/30°C)
FH57	None	73	70	1.04	3.1	6.7	2.2
FH57	pJC701	99	95	1.04	3.0	6.5	2.2
FH57	pJC703	92	61	1.52	3.1	3.5	1.1
AB468[d]	None	68	45	1.52	3.0	3.2	1.1
AB468	pJC701	90	57	1.56	3.1	3.5	1.1
AB468	pJC703	77	49	1.56	2.7	3.3	1.2

[a] See Table 1 for genotypes of the strains.
[b] See Fig. 1 for structure of the plasmids.
[c] The cells were grown exponentially at 30°C in glucose-amino acid medium. Samples for chemical DNA determination were taken before and 2 h after a shift to 39°C.
[d] A single experiment. All other values are averaged over two independent experiments.

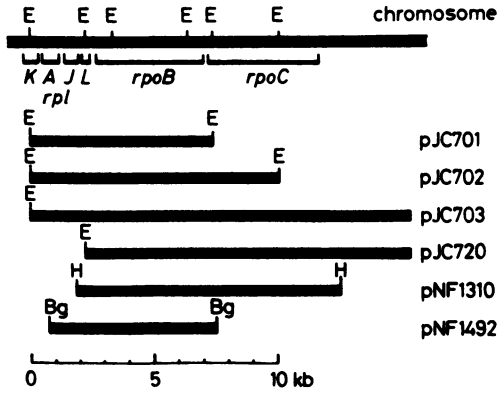

FIG. 1. Structure of plasmids used in complementation analysis. The genetic structure of the chromosome in the *rpoB,C* region is indicated together with the *Eco*RI restriction enzyme pattern (E). The chromosomal DNA carried by the ColE1-derived pJC plasmids and the pBR322-derived pNF plasmids is redrawn from Fiil et al. (10). The other restriction enzymes used to generate the respective plasmids are indicated: H, *Hin*dIII; B, *Bgl*II. A kilobase (kb) scale is shown for reference.

merase was also prepared from derivatives of strain FH57 containing plasmid pJC701 or pJC703 (Fig. 1). Due to the copy number of ColE1, we would expect a 15-fold excess of plasmid-encoded over chromosomally encoded proteins. Both plasmids carry the normal *rpoB,C* promoter (20). pJC701 carries the intact *rpoB* gene and should therefore provide the wild-type β subunit to the RNA polymerase. pJC703 carries both *rpoB* and *rpoC* and should replace most of the mutant RNA polymerase β and β′ subunits. Supplementation with the wild-type β subunit did not restore a wild-type RNA-synthesizing activity (Fig. 2D); supplementation with both wild-type β and β′ subunits restored the wild-type RNA-synthesizing activity of the extract (Fig. 2C). These experiments indicated that the in vitro temperature sensitivity of the

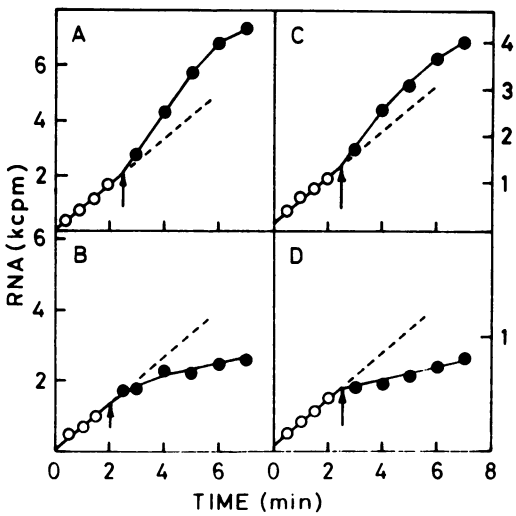

FIG. 2. RNA synthesis in partially purified RNA polymerase extracts at 30 and 40°C of (A) strain AB468 (wild type), (B) strain FH57 (*rpoC907 rpoB906*), (C) strain FH57 (*rpoC907 rpoB906*) carrying pJC703(*rpoC*$^+$ *rpoB*$^+$), and (D) strain FH57 (*rpoC907 rpoB906*) carrying pJC701 (*rpoB*$^+$). RNA synthesis was measured as the incorporation of [^{3}H]UTP into TCA-precipitable material. The arrow indicates the time at which the assay mixture was transferred to 40°C. (○ and ●), Accumulated RNA at 30 and 40°C, respectively.

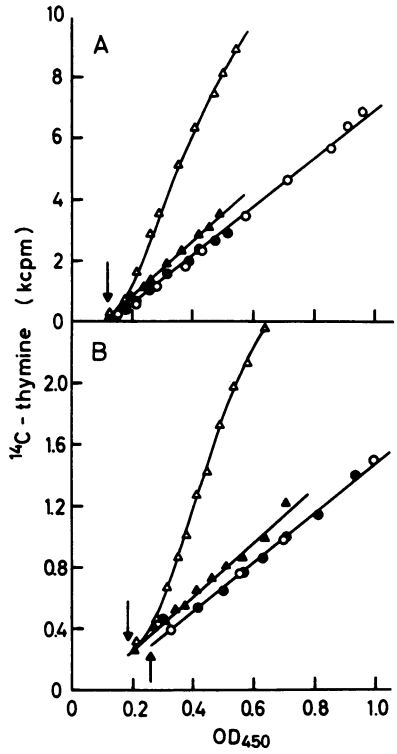

FIG. 3. *rpoB,C* mutations cause overproduction of DNA at 39°C. Two strains carrying the *rpoB,C* mutations in different genetic backgrounds and the corresponding wild-type strains were grown exponentially at 30°C in glucose-minimal medium supplemented with thymine and Casamino Acids. [^{14}C]thymine was added, and a few minutes later a part of the cultures was shifted to 39°C. (A) The original mutant strain CP387 and its parent, CP385. (B) Strains TC98 (*rpoB906 rpoC907*) and TC92 (*rpoB*$^+$ *rpoC*$^+$). (▲, ●) Cultures at 30°C; (△, ○) cultures at 39°C. (▲, △) RNA polymerase mutant; (●, ○) parent or isogenic wild-type strain.

mutant RNA polymerase is dependent on the mutational alteration in the *rpoC* gene.

Complementation and reversion of the DNA replication phenotype. The experiments to study the regulation of DNA replication in the mutant (Fig. 3B, 4, and 5B) were carried out with strain TC98, containing both *rpoC907* and *rpoB906*. However, the complementation analysis (Table 4) strongly suggested that the DNA replication phenotype of a strain carrying these two *rpo* mutations (strain FH57) is set by the presence of the *rpoC907* allele. At 39°C, strain FH57(pJC701) showed slow growth and a high DNA/mass ratio relative to those at 30°C. In strain FH57(pJC703), both the growth rate and the DNA/mass ratio were the same independent of the temperature, as was observed for the wild-type control strains with or without plasmids. It should be emphasized that pJC701 contributes only the wild-type β subunit of the RNA polymerase, whereas pJC703 contributes both the β and β′ subunits (cf. Fig. 1) and that pJC701 could complement the *dnaA46* suppressor activity of the *rpoB906* mutation (Table 3).

Furthermore, spontaneous temperature-resistant revertants of strain TC98 (*rpoB906 rpoC907*) all showed simultaneous reversion of the other phenotypes attributed to the *rpoC907* mutation, i.e., increase in DNA/mass ratio after a shift to 39°C, slow growth rate at 39°C, and filament formation at high temperature.

Regulation of DNA replication in strains carrying *rpoC907*. DNA synthesis was followed through temperature shifts from 30 to 39°C. Figure 3A shows the response of the original mutant strain CP387, carrying the three mutations described above. Fig. 3B shows the response of strain TC98, which only carries the *rpoC907* and *rpoB906* alleles. The corresponding isogenic wild-type strains were included as controls. [^{14}C]thymine incorporation into the DNA was plotted against mass (OD_{450}). The slope of the curve (ΔDNA/ΔOD) was not affected by the temperature shift in the wild-type strains. In contrast, this slope increased soon after the shift in both strains carrying the mutant RNA polymerase. After about one mass doubling at 39°C, the DNA/mass ratio of the mutant strains reached a new constant value that was more than twice the preshift value, which was very close to that of the control strains. The increased DNA/mass ratio was still observed after more than 20 mass doublings at 39°C.

Figure 4 shows a temperature shift experiment with strain TC98 in which the origins, total DNA, and OD_{450} (mass) were followed. The

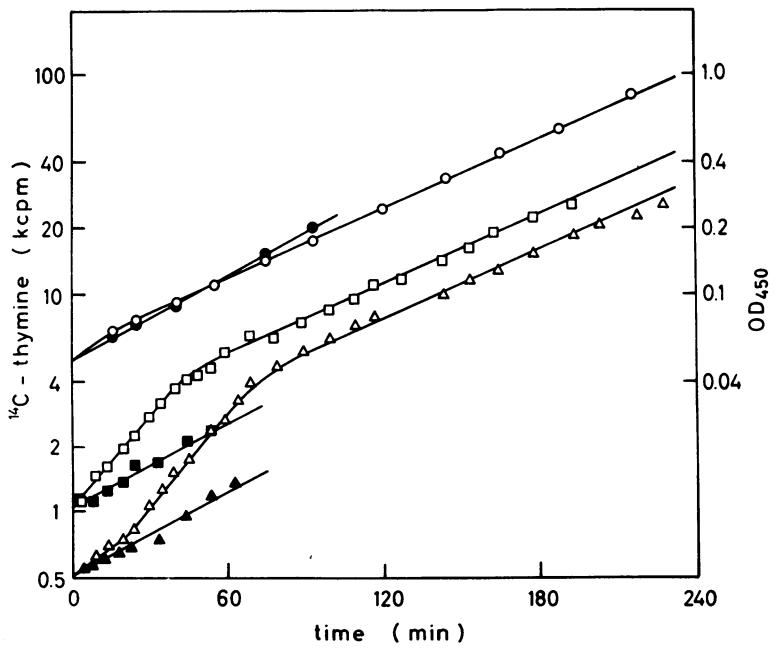

FIG. 4. Effect of the *rpoB906 rpoC907* alleles on DNA synthesis during a shift from 30 to 39°C. Cells of strain TC98 (*rpoC907 rpoB906*) were grown exponentially at 30°C in glucose-Casamino Acids medium supplemented with [^{14}C]thymine. At time zero a part of the culture was shifted to 39°C. Solid symbols, Cells continuing growth at 30°C. Open symbols, Cells shifted to 39°C. Circles, OD at 450 nm. Triangles, Total DNA measured by [^{14}C]thymine incorporation. Squares, Total DNA in cells incubated in chloramphenicol (200 μg/ml) for 180 min after sampling (proportional to origin concentration), measured by [^{14}C]thymine incorporation.

amount of origins was estimated as the amount of DNA accumulated in small samples drawn from the culture and treated with chloramphenicol. The amount of DNA will be proportional to the number of origins (12), assuming that little or no initiation can occur in the absence of protein synthesis and that ongoing rounds of replication are terminated. DNA synthesis ceased within 2 h after chloramphenicol addition regardless of the growth temperature in both the parent and mutant strains (data not shown). Inhibition of initiation by the addition of rifampin resulted in the same final amount of DNA as did the addition of chloramphenicol.

The rate of mass increase dropped by about 20% soon after the shift (this was also the case for both RNA and protein synthesis [data not shown]), but the rate of initiation (origin accumulation) doubled immediately after the shift. After 60 min (one mass doubling), the initiation rate was readjusted so that origins accumulated in balance with the mass increase of the culture. This new origins/mass ratio was maintained when growth became balanced at 39°C (Fig. 4 and 5B). The acceleration of DNA chain elongation that might be expected from the temperature shift seemed to be delayed by about 15 min. In this period there was only a slight increase in the rate of DNA accumulation, probably due to the addition of new replication forks. When the culture reached the new balanced state, the replication time was normal as estimated from measurements of residual DNA synthesis in the absence of protein synthesis (Fig. 4).

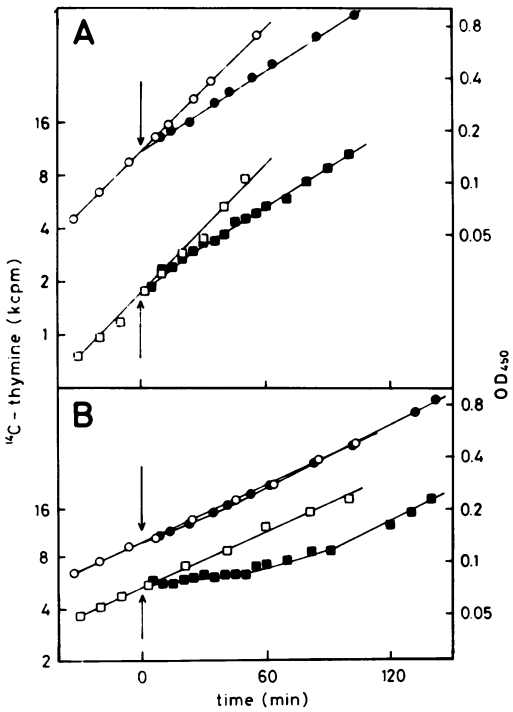

FIG. 5. Effect of the *rpoB906 rpoC907* alleles on the initiation of DNA replication during a shift from 39 to 30°C. Cells of (A) strain TC92 (*rpoB*$^+$ *rpoC*$^+$) and (B) strain TC98 (*rpoB906 rpoC907*) were grown exponentially at 39°C in glucose-Casamino Acids medium supplemented with [^{14}C]thymine. At time zero part of the culture was shifted to 30°C. Open symbols, Cells continuing growth at 39°C. Solid symbols, Cells shifted to 30°C. Circles, OD at 450 nm. Squares, Origins (total DNA in cells incubated in chloramphenicol [200 μg/ml] for 180 min after sampling, measured as [^{14}C]thymine incorporation).

Figure 5 shows a shift in temperature from 39 to 30°C with the mutant strain TC98 (Fig. 5B) and the corresponding wild-type strain TC92 (Fig. 5A). The cultures showed balanced growth at 39°C, and mass (OD) and origins, measured as described above, were followed during the shift. In the experiment with the wild-type strain, both the rate of mass increase and the rate of accumulation of origins decreased about 35%, as expected, after the temperature shift (Fig. 5A). The mutant strain showed the characteristic reduced growth rate at 39°C, and the rate of mass increase was little affected by the temperature shift to 30°C. Initiation of DNA replication (accumulation of origins), however, came to an almost complete stop within 5 min after the shift and was restarted only when the origins/mass ratio characteristic of growth at 30°C was approached.

The *rpoC907* mutation had an effect on the general growth properties of the cells, probably due to a primary influence on RNA synthesis. At 30°C, the rate of mass increase was reduced about 20% relative to the parent strain regardless of the growth medium. At 39°C, the richer the medium, the greater the reduction in growth rate was: 35% reduction in glycerol, 40% reduction in glucose-minimal medium, and 55% in glucose-Casamino Acids medium. However, the two- to threefold increase in the origins/mass ratio at 39°C was observed with all growth media.

DISCUSSION

Normally in bacteria the origin concentration—the copy number of the chromosome—is nearly constant and only slightly dependent on the growth rate (9, 21, 23). The mutant strain CP387 carries a mutation in the RNA polymerase that leads to an increased copy number of the chromosome at high temperature. At 39°C the chromosomal origin concentration (origins/mass ratio) was more than twofold that at 30°C, where it was the same as that of the parent.

The mutant was found to carry two mutational alterations in the RNA polymerase: the *rpoC907*

mutation, which caused both temperature sensitivity of growth and the temperature-dependent chromosome copy number increase, and the *rpoB906* mutation, which suppressed the temperature sensitivity caused by the *dnaA46* mutation. These two mutations were allocated to the *rpoC* and *rpoB* genes, respectively, by complementation with plasmids (Fig. 1, Tables 3 and 4) carrying various amounts of chromosomal DNA from the *rpoB,C* region.

The activity of partially purified RNA polymerase from a strain carrying these two *rpo* mutations was inhibited at high temperature (40°C). This inhibition was relieved by in vivo complementation with the β and β′ subunits together, but not with β alone. This shows that the mutated β′ subunit renders the RNA polymerase temperature sensitive in vitro. Purified RNA polymerase from the mutant shows the same temperature inhibition as does the partially purified preparation (A. Ishihama and F. G. Hansen, unpublished data).

Our physiological experiments were carried out with strains containing both the *rpoC907* and *rpoB906* mutations. The characteristic phenotype of the mutant—a twofold increase in origin concentration at 39°C—was complemented by plasmid pJC703, which supplied both the wild-type β and β′ subunits of the RNA polymerase, but not by pJC701, which supplied only the β subunit (Table 4). This shows that the *rpoC907* mutation is necessary for the observed change in the initiation control. In a strain carrying the *dnaA46* and *rpoB906* mutations, the excess of pJC701-encoded wild-type β subunit over the chromosomally encoded mutant β subunit was sufficient to eliminate the *dnaA* suppressor phenotype (Table 3). Thus, the lack of complementation of the increased DNA/mass ratio at 39°C in the *rpoC907 rpoB906* strain suggests that the *rpoB906* mutation is dispensable for expression of the DNA phenotype conferred by the *rpoC907* mutation.

The mutant in this study showed only moderate changes in growth rate after a shift from 30 to 39°C, ranging from a 20% decrease in rich medium to a 20% increase in poor medium. The growth rate of the wild-type strain increased 50 to 70% regardless of the growth medium. This indicates that at 39°C growth was limited by the mutant RNA polymerase, especially in medium normally supporting fast growth. The specific effect of the *rpoC907* mutation on the initiation of DNA replication was independent of the growth medium, and the relatively small changes in growth rate could not by themselves result in any significant change in the origin concentration, which is normally independent of the growth rate (9). In the mutant strain, new origins were formed at a rate far exceeding the rate of mass increase of the culture immediately after the shift, indicating that the mutant had escaped the normal coupling between increase in mass and initiation of replication. After about one mass doubling, this coupling was restored but with a new, decreased cell mass per chromosomal origin less than half the preshift value. Within 5 min after a shift from 39 to 30°C (permissive temperature), the initiation of DNA replication (formation of new origins) was almost completely inhibited in the mutant strain. This inhibition was maintained for about one mass doubling, after which the normal origin concentration was approached. These alterations in the normal coupling between mass increase and initiation of DNA replication shown by the mutant in the temperature shift experiments indicate that the *rpoC907* mutation specifically affects the control of initiation of DNA replication in addition to its effect on the general growth properties.

From our current knowledge about the initiation of DNA replication—the requirement for (i) de novo protein synthesis (15, 19), possibly in the form of specific initiator protein(s), and (ii) a transcriptional step (14)—we can imagine two different ways to explain how a mutated RNA polymerase might cause the observed alterations in the control of initiation of DNA replication: (i) a temperature-dependent change in the rate of synthesis of the mRNA for an initiation control protein (this could be either an inhibitor, as proposed by Pritchard [22], or a specific initiator protein limiting initiation, for instance the *dnaA* protein [12]); or (ii) a temperature-dependent change in the transcriptional step in the initiation process, most likely mediated through a changed interaction between the RNA polymerase and an initiation control protein.

We cannot at present discriminate between these possibilities. We hope that the future study of this mutant will shed some light on the system controlling the initiation of chromosome replication, a process of which we still know very little.

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