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Mode of Initiation of Constitutive Stable DNA Replication in RNase H-Defective Mutants of *Escherichia coli* K-12

KASPAR VON MEYENBURG,[1,2]† ERIK BOYE,[3] KIRSTEN SKARSTAD,[3] LUUD KOPPES,[1]‡ AND TOKIO KOGOMA[1,2,4]*

Department of Microbiology, The Technical University of Denmark, DK-2800 Lyngby-Copenhagen, Denmark[1]; Department of Biophysics, Institute for Cancer Research, Montebello, 0310 Oslo, Norway[3]; and Department of Biology[2] and Department of Cell Biology, School of Medicine,[4] University of New Mexico, Albuquerque, New Mexico 87131*

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The alternative pathway of DNA replication in *rnh* mutants of *Escherichia coli* can be continuously initiated in the presence of chloramphenicol, giving rise to constitutive stable DNA replication (cSDR). We conducted a physiological analysis of cSDR in *rnh-224* mutants in the presence or absence of the normal DNA replication system. The following results were obtained. (i) cSDR allowed the cells to grow in the absence of the normal replication system at a 30 to 40% reduced growth rate and with an approximately twofold-decreased DNA content. (ii) cSDR initiation was random with respect to time in the cell cycle as well as choice of origins. (iii) cSDR initiation continued to increase exponentially for more than one doubling time when protein synthesis was inhibited by chloramphenicol. (iv) cSDR initiation was inhibited during amino acid starvation in stringent (*relA*$^+$) but not in relaxed (*relA1*) strains, indicating its sensitivity to ppGpp. (v) cSDR initiation was rifampin sensitive, demonstrating that RNA polymerase was involved. (vi) cSDR functioned in *dnaA*$^+$ *rnh-224* strains parallel to the normal *oriC*$^+$ *dnaA*$^+$-dependent chromosome replication system.

The stable DNA replication (SDR) phenotype of constitutive SDR (cSDR) mutants (formerly *sdrA*) has been demonstrated to be due to a mutation in the *rnh* gene resulting in lack of RNase H activity (28). The SDR phenotype becomes manifest under the following two situations. (i) In *rnh* mutants, DNA replication continues in the presence of chloramphenicol for an extended period (9) (Fig. 1B), whereas it ceases in the wild-type cells after a short period (Fig. 1A) which reflects the time for ongoing replication forks to terminate (20, 24). (ii) The introduction of an *rnh* mutation renders *dnaA*(Ts) strains viable at 42°C (15) and even allows for insertional inactivation of the *dnaA* gene or deletion of the origin of chromosomal replication, *oriC* (15).

It is therefore pertinent to ask what the characteristics were of this alternative chromosomal replication pathway which operates, in the absence of RNase H, independently of the two main elements of normal replication initiation, DnaA protein and *oriC*. It has already been demonstrated that there are at least four different alternative initiation sites (origins) or regions, termed *oriK*, from which cSDR originates in *rnh* mutant cells (7). Also, it has been shown that initiation of cSDR is *recA*$^+$ dependent (12, 34). Here we present a physiological analysis of cSDR in *rnh* mutant strains in the presence or absence of the normal replication system. This analysis is based on determination of DNA content and cell size, replication pattern by density shift technique, and DNA synthesis pattern after chloramphenicol addition, rifampin treatment, and amino acid starvation.

* Corresponding author.
† Present address: Chr. Hansen's Laboratorium A/S, DK-1250 Copenhagen, Denmark.
‡ Present address: Department of Microbiology, Biomedical Center, University of Uppsala, S-751 23 Uppsala, Sweden.

MATERIALS AND METHODS

Bacterial strains. The *Escherichia coli* K-12 strains constructed and used in this study are described in Table 1. P1 transductions were done by the method of Lennox (21).

Growth conditions, amino acid starvation, and radioactive labeling of DNA. The growth medium used was the phosphate-based mineral salt medium A + B (6) supplemented with 0.2% glucose, required amino acids at 20 μg/ml, uracil (15 μg/ml), and thiamine hydrochloride (2 μg/ml). Vitamin-free Casamino Acids (Difco Laboratories, Detroit, Mich.) were added at 0.5% to enrich the medium. Single colonies of the various strains were inoculated in several dilutions of 10 ml of the respective growth medium and incubated at 30 or 41°C for at least 20 h with aeration. These inoculum cultures were diluted into fresh medium 1:20 to 1:50 to an A_{450} of 0.02 to 0.04. Growth was followed by measuring A_{450} (36).

Amino acid starvation conditions were attained by decreasing the tryptophan concentration to 0.3 μg/ml; at an A_{450} of approximately 0.2, protein synthesis ceased abruptly owing to complete consumption of the required amino acid tryptophan. Chloramphenicol and rifampin were added at 150 and 200 μg/ml, respectively, to inhibit protein or RNA synthesis.

For the determination of DNA and RNA concentration, *pyrB* mutant strains were radioactively labeled with [^{14}C]uracil (0.7 μCi/μmol) during exponential growth or various treatments. For determination of DNA, samples (0.4 ml) were removed at intervals of 10 to 30 min and added to 0.8 ml of 1 N NaOH on ice. After hydrolysis of RNA by incubation for 2 h at 37°C (36), the alkali-stable DNA was precipitated by the addition of 2 ml of ice-cold 10% trichloroacetic acid and collected on Whatman GF glass filters (25-mm diameter), and the radioactivity was determined by scintillation counting (36). Parallel samples of 0.4 ml were taken into 1 ml of 10% trichloroacetic acid, and the precip-

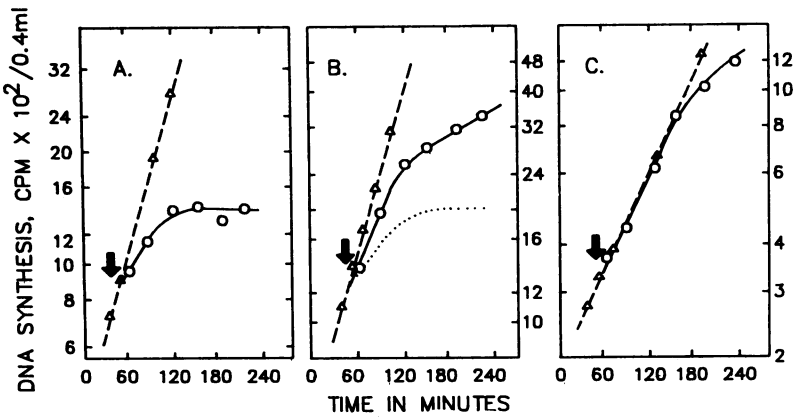

FIG. 1. DNA synthesis in the presence and absence of chloramphenicol. Cells of the *rnh*$^+$ *dnaA*$^+$ strain CM3490 (A), the *rnh-224 dnaA*$^+$ strain CM3591 (B), and the *rnh-224 dnaA850*::Tn*10* strain M3847 (C) were exponentially grown in glucose-Casamino Acids medium at 41°C and labeled with [^{14}C] uracil at 41°C. Chloramphenicol at 150 μg/ml was added to a portion of the cultures at the time indicated by the arrow. DNA synthesis in the presence (○) or absence (△) of chloramphenicol was determined by measuring radioactivity in alkali-stable material as described in the text.

itated cells were collected for determination of radioactivity, which in these samples represents RNA plus DNA. Radioactivity in RNA was determined by subtracting the counts in DNA determined as described above. Absolute values for RNA and DNA were calculated based on the specific activity of [^{14}C]uracil in the medium and the mole percentage of pyrimidine bases in RNA and DNA, respectively (26). As a control, RNA concentrations were also determined by measurement of UV absorption in alkaline cell hydrolysates (36).

Microphotography. Microphotography was performed as described earlier (40) on cells fixed with 0.5% formaldehyde.

Flow cytometry. Bacteria were fixed and stained for flow cytometry as described previously (30). Scattered light and DNA-associated fluorescence from individual cells were measured with a laboratory-built flow cytometer (31, 33). The data were recorded as two-parameter (i.e., DNA and light scatter) histograms which could be integrated along one axis to give one-parameter DNA or light scatter histograms. The light scattered by each cell was proportional to protein content (4).

Determination of chromosomal replication pattern by density shift of [^{3}H]thymidine pulse-labeled DNA. Strains AQ685 and DK249 were each grown in 25 ml of heavy MOPS (morpholinepropanesulfonic acid) medium (27) containing 2.0 mM [^{13}C]glucose and 1.3 mM $^{15}NH_4Cl$, supplemented with methionine, tryptophan, and histidine (20 μg of each per ml) and proline (40 μg/ml). The doubling time of AQ685 was 75 min at 30°C and that of DK249 was 67 min at 37°C. When the A_{450} reached 0.5, the cultures were pulse-labeled with 2 μCi of [^{3}H]thymidine (47 Ci/mmol) per ml for 1/10th of the doubling time. Then 60 ml of prewarmed MOPS medium was added giving a final concentration of 0.4% [^{12}C]glucose, 20 mM $^{14}NH_4Cl$, 8 μM thymidine, and 80 μM uridine. At successive times after the shift or chase, 10-ml samples were taken; the cells were washed and lysed, and the DNA was sheared by drawing it five times through a 21G2 (50/8) needle. Finally the ^{3}H-labeled DNA fragments in each sample were separated into heavy-density (unreplicated) and hybrid-density (re-replicated) components by equilibrium centrifugation in CsCl as described previously (16).

RESULTS

Effects of *rnh-224* mutation on growth, DNA content, and cell size. The comparison of the cSDR pattern with normal replication in *rnh* mutant strains was based on the analysis of growth and DNA replication in sets of strains which differed

TABLE 1. *E coli* K-12 strains

Strain	Relevant genotype	Reference or construction
TC743	*rpoB*$^+$ *metB*$^+$	15
DK249[a]	*rnh-224 rpoB340 metB1 dnaA850*::Tn*10*	15
DK322[b]	*pyrB zjg*::Tn*5*	*pyrB* and *zjg*::Tn*5* are 50% linked (Lars Boe)
AQ685[a]	*rnh*$^+$ rpoB340 metB1 dnaA$^+$	15
AQ2561[c]	*relA1 zga*::Tn*10*	*relA1* and *zga*::Tn*10* are 70% linked (this work)
CM3438[a]	*rnh*$^+$ *dnaA*$^+$ *rpoB*$^+$ (as AQ2696)	13
CM3440[a]	*rnh-244 dnaA*$^+$ *rpoB*$^+$	13
CM3442[a]	*rnh-224 dnaA46 tnaA600*::Tn*10 rpoB*$^+$	13
CM3452[a]	*rnh-224 dnaA850*::Tn*10 rpoB*$^+$	P1 (TC743) × DK249, select Met$^+$, screen Rif^s
CM3590[a]	*rnh*$^+$ *dnaA*$^+$ *pyrB zjg*::Tn*5*	P1 (DK322) × CM3438, select Kan^r, screen Ura$^-$
CM3591[a]	*rnh-224 dnaA*$^+$ *pyrB zjg*::Tn*5*	P1 (DK322) × CM3440, select Kan^r, screen Ura$^-$
CM3592[a]	*rnh-224 dnaA46 pyrB zjg*::Tn*5*	P1 (DK322) × CM3442, select Kan^r, screen Ura$^-$
CM3847[a]	*rnh-224 dnaA850*::Tn*10 pyrB zjg*::Tn*5*	P1 (DK322) × CM3452, select Kan^r, screen Ura$^-$
AQ3405[a]	*rnh-224 dnaA46 pyrB relA*$^+$	Tet^s derivative of CM3592 (this work)
AQ3497[a]	*rnh-224 dnaA46 pyrB relA1 zga*::Tn*10*	P1 (AQ2561) × CM3592, select Tet^r, screen Rel$^-$

[a] The remaining genotype is F$^-$ *trpA9605 his-29 argH metD88 deoB* or *C*.
[b] The remaining genotype is: F' *ilv asnA*::Tn*10 asnB32 relA1 spoT1 lysA*.
[c] The remaining genotype is: Hfr *thi galE fuc* Δ(*att-bio*) *deoA103 deoC lysA cytR upp udp*.

TABLE 2. Growth characteristics of *rnh dnaA* mutants[a]

Strain (relevant genotype)	Growth temp (°C)	Origin usage		Doubling time (*Td*) (min)	DNA conc ($\mu g/A_{450} \cdot ml$)	Cell size[c] ($A_{450}/10^8$ cells)
		oriC	*oriK*			
CM3590 (*dnaA*$^+$ *rnh*$^+$)	30	+	−	58[b]	4.3	0.32 ± 0.02
CM3591 (*dnaA*$^+$ *rnh-224*)	30	+	+	66[b]	4.6	0.29 ± 0.02
CM3592 (*dnaA46 rnh-224*)	30	+	+	75	3.3	0.5 ± 0.1
	41	−	+	57	2.15	1.13 ± 0.07
CM3847 (*dnaA*::Tn*10 rnh-224*)	30	−	+	105	2.3	0.7 ± 0.04
	41	−	+	65	1.8	1.65

[a] Determined during balanced exponential growth in AB-glucose-Casamino Acids medium.
[b] *Td* for CM3590 and CM3591 at 41°C was 36 min.
[c] Determined for the otherwise isogenic *pyrB*$^+$ strains CM3438, CM3440, CM3442, and CM3452. Average of two determinations.

with respect to the *dnaA* and *rnh* alleles. A typical set of such strains was CM3438, CM3440, CM3442, and CM3452 which were *dnaA*$^+$ *rnh*$^+$, *dnaA*$^+$ *rnh-224*, *dnaA46 rnh-224*, and *dnaA850*::Tn*10 rnh-224*, respectively. For the analysis of DNA synthesis we found it most advantageous to use uracil-requiring derivatives (*pyrB*) of these strains (CM3590, CM3591, CM3592, and CM3847, respectively) to follow quantitatively the incorporation of [^{14}C]uracil into alkali-stable material (viz. DNA) (see Materials and Methods). The steady-state growth data for these strains growing exponentially in glucose minimal medium enriched with Casamino Acids at 30 and 41°C are presented in Table 2. Strains CM3590 and CM3591 had very similar DNA contents and average cell sizes (A_{450} per 10^8 cells) at 30°C. The latter strain, which is *rnh*, actually appeared to have a slightly increased DNA content and decreased average cell size. In strain CM3592 at 41°C, at which temperature the normal replication initiation system was inhibited owing to the inactivation of the DnaA46 protein, the DNA content decreased by 50%, growth rate ($1/Td$, where *Td* is the doubling time) diminished by 35% relative to the wild-type CM3590 (36 min), and cell size drastically increased (Table 2). The respective values for strain CM3847, in which the *dnaA* gene is disrupted by a Tn*10* insertion, were rather similar at both 30 and 41°C; the growth rate was decreased by 45% at both temperatures, yet DNA content was slightly lower and average cell size even larger than for CM3592 at 41°C. Similar differences in DNA content, cell size, and growth rate were found between these strains when they were grown in glucose minimal medium without Casamino Acids supplement (data not shown).

Calculation of the rate of DNA synthesis per unit cell mass [$dD/dt \times 1/A_{450}$ = (DNA amount/A_{450}) × $1/Td \times \ln 2$] revealed that it decreased three- to fourfold in the *rnh* mutant strains (CM3592 at 41°C and CM3847 at 30 and 41°C) compared with that of the CM3590 and CM3591 strains at the respective temperatures; thus, the cSDR system apparently operates with a considerably lower efficiency than the normal replication system.

Phase-contrast microscopy (Fig. 2A to E) supported the data on DNA content and average cell size (Table 2). The presence of the *rnh* allele in the *dnaA*$^+$ strain CM3438 (Fig. 2B) or the *dnaA46* strain CM3440 at 30°C (Fig. 2C) did not appear to affect cell size and cell morphology significantly. For the strains which solely operate with cSDR, however, the cells drastically increased in size; yet, not all cells in the populations were elongated (Fig. 2D and E), yielding a rather heterogeneous overall cell-size distribution. This suggested to us that the cell growth and DNA replication in exponentially growing cSDR (*rnh*) strains were considerably less well coupled than in *dnaA*$^+$ strains. This led us to investigate, on the one hand, the mass and DNA distribution by flow cytometry and, on the other hand, the pattern of DNA re-replication by a density shift experiment.

Cell mass and DNA distribution and DNA replication pattern in rnh mutant strains. By dual-parameter flow cytometry we obtained three-dimensional profiles for the mass and DNA distribution in the populations. The *dnaA*$^+$ strain CM3590 (Fig. 2F) had the narrow frequency distribution typical of exponentially growing wild-type *E. coli* K-12 cells (32). Strain CM3592 (*rnh dnaA46*) at 30°C exhibited a considerably broader distribution (Fig. 2G), similar to the one for an *rnh*$^+$ *dnaA46* strain (data not shown). At 41°C the distribution for the *rnh dnaA46* strain became even broader and more complex (Fig. 2H). There are indications for two discrete subpopulations in this distribution with peaks of cells with one and two chromosome equivalents, respectively. The broad distribution (Fig. 2H) indicates that there existed only a very loose correlation between cell mass and DNA content in cSDR cells. The DNA content appeared to vary considerably more for a given cell mass (light scatter) than it did in the wild type (Fig. 2F).

Information on the pattern of DNA replication can also be obtained by a density shift transfer experiment since such experiments allow one to determine how much time elapses between successive rounds of replication (16). In wild-type cells, any freshly replicated segment of chromosomal DNA is re-replicated fairly precisely one doubling time later (Fig. 3) (16, 17, 38), characteristic for a well-timed replication initiation system. In contrast, re-replication of freshly replicated DNA in the *rnh-224 dnaA*::Tn*10* strain, DK249, which lacks the active DnaA protein was completely different (Fig. 3). This pattern of re-replication, i.e., the pattern of shift of ^{3}H label from the heavy-heavy to the heavy-light DNA fraction, is indicative of the lack of orderly timing of initiation (see reference 16 for theory). It could be envisaged simply to be due to a random choice of one origin among the several *oriK* sites (7) which was fired at a fixed time in a cell cycle. However, if cSDR initiation at a randomly chosen origin was timed at about one doubling time interval, a considerably less random re-replication curve would have been expected. We infer that initiation of cSDR in *rnh dnaA* double mutants is random with respect to both the time and the choice of initiation sites.

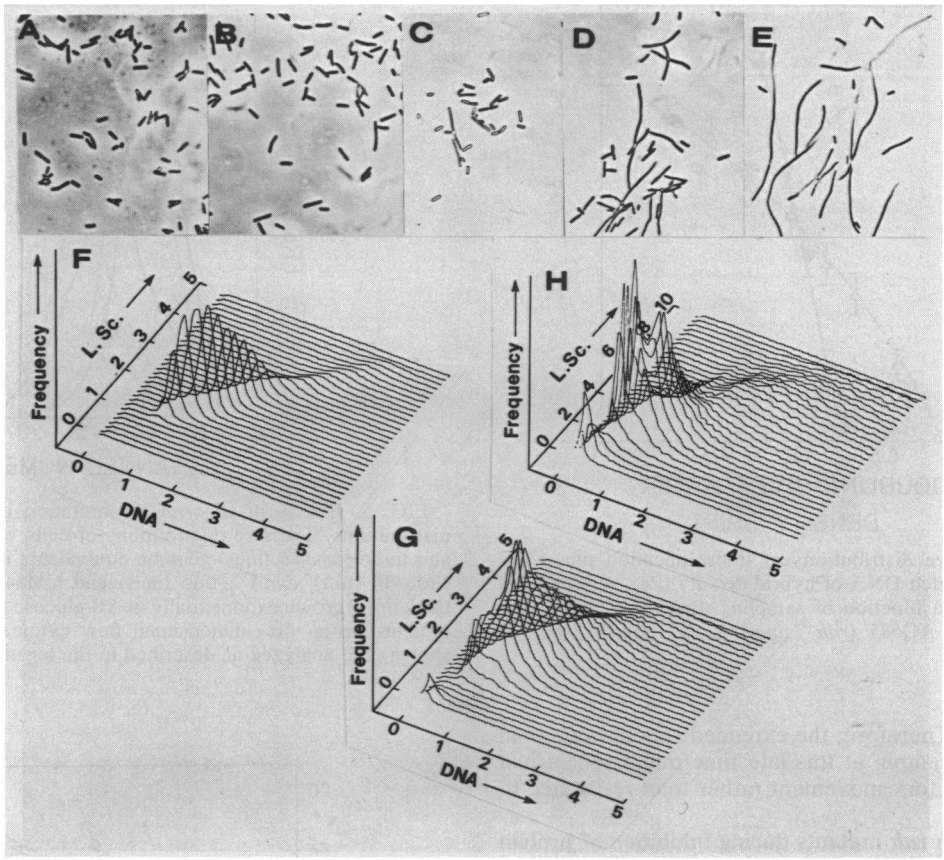

FIG. 2. Microphotographs (A to E) and DNA and light scatter histograms (F to H). Cells of CM3438 (*rnh*$^+$ *dnaA*$^+$) growing exponentially in AB-glucose-Casamino Acids medium at 41°C (A), CM3440 (*rnh-224 dnaA*$^+$) at 41°C (B) CM3442 (*rnh-224 dnaA46*) at 30°C (C) CM3442 at 41°C (D) and CM3452 (*rnh-224 dnaA850*::Tn*10*) at 41°C (E) were photographed under a microscope (40). Populations of exponentially growing cells (Casamino Acids medium) of CM3590 (*rnh*$^+$ *dnaA*$^+$) at 41°C (F) CM3592 (*rnh-224 dnaA46*) at 30°C (G), and CM3592 at 41°C (H) were also analyzed by flow cytometry as described in Materials and Methods. The DNA and light scatter (L.Sc.) axes are arbitrary. One chromosome equivalent corresponds to approximately 0.59 with the arbitrary scale. Note the different scale on the light scatter axis in panel H owing to a different amplification of the light scatter signal.

A closer analysis of the DNA mass distributions obtained by flow cytometry (Fig. 4 and 5) supports our conclusion of randomness of initiation of cSDR. Notably, a large fraction of CM3592 cells grown at 41°C with two genome equivalents had cell sizes equal to and even considerably larger than that of three-genome-equivalent cells of the *dnaA*$^+$ *rnh*$^+$ strain (Fig. 4A and B). The same was true for the one- and three-genome-equivalent cells. On the other hand, when comparing, for example, the two- or three-chromosome cell-size distributions (Fig. 4A and B), we consistently found a small fraction of cells in cultures of strain CM3592 grown at 41°C which were smaller in size for a given amount of DNA than the wild type, representing cells with too much DNA. This suggests that there were cells in the *rnh dnaA* double mutant cell population in which successive rounds of cSDR were initiated with time intervals shorter than the doubling time. Conversely, the finding of large cells with too little DNA as noted above is an indication of cells in which initiation of successive rounds of replication was delayed considerably. Cells with a normal DNA content in these populations represent those in which successive rounds of replication on the average occurred with a frequency similar to that in the wild-type. The above arguments are of course only valid if the *rnh* mutation had no direct effect on cell division. Such a possibility appears to be ruled out, since the *rnh-224 dnaA*$^+$ strain (CM3440) did not exhibit any abnormality of cell size distribution, except for the presence of a fraction of cells with too much DNA as visualized by the comparison of the size distribution (light scatter) of *dnaA*$^+$ *rnh-224* cells with two or three genome equivalents with those of strain CM3438 (*dnaA*$^+$ *rnh*$^+$) (Fig. 5).

Initiation of cSDR is rifampin sensitive. Initiation of normal *oriC*$^+$ *dnaA*$^+$-dependent replication requires participation of active RNA polymerase, as the rifampin sensitivity of the initiation process in vivo had shown (18, 25, 39). After the addition of rifampin, DNA synthesis ceased gradually in a wild-type strain, coming to a standstill approximately 60 to 80 min later. These runout kinetics reflect the completion of ongoing rounds of replication in individual cells. Rifampin treatment of *rnh* mutant strains resulted in a similar runout of DNA synthesis irrespective of whether the normal initiation pathway was inactivated or not (Fig. 6). The amount of residual DNA synthesis, as the percentage of DNA present at the time of inhibition, was the same in the *rnh-224 dnaA46* strain at 41°C (Fig. 6, R_0 curve) as in the wild-type CM3590 after chloramphenicol (Fig. 1A) or rifampin (data not shown) treatment.

It seems as though the cSDR initiations became rifampin resistant after 80 to 100 min of treatment with chloramphenicol (Fig. 6, R_{80} curve) (9). However, the rate of cSDR started slowing down markedly even in the absence of rifampin 80 to 100 min after inhibition of protein synthesis

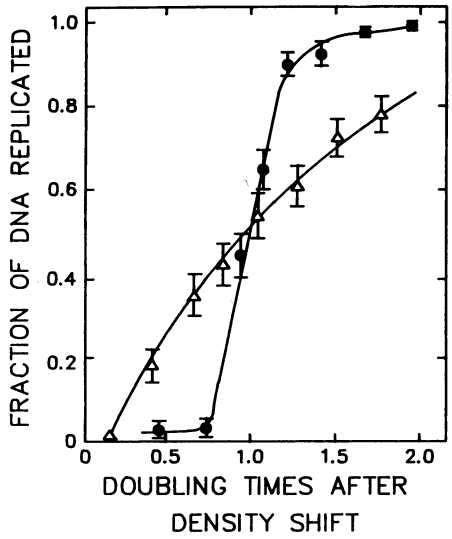

FIG. 3. Cumulative distributions of interreplication time. The fraction of pulse-labeled DNA of hybrid density (i.e., re-replicated DNA) is plotted as a function of sampling time after the density shift. Symbols: ●, AQ685 (*rnh*$^+$ *dnaA*$^+$); △, DK249 (*rnh-224* dnaA850::Tn*10*).

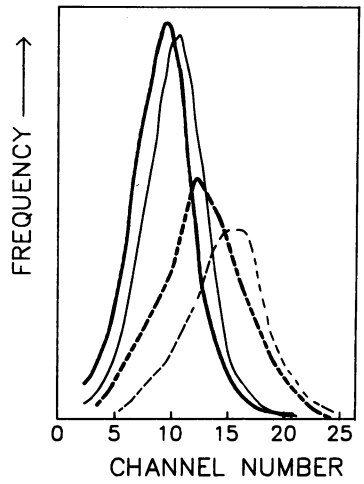

FIG. 5. Effects of the *rnh-224* mutation on relative cell size distributions. The size distributions of cells with two (solid lines) and three (dashed lines) genome equivalents in the populations of CM3438 (*rnh*$^+$ *dnaA*$^+$; thin lines) and CM3440 (*rnh-224 dnaA*$^+$; thick lines) grown exponentially in AB-glucose medium at 30°C were obtained from three-dimensional flow cytometry histograms (not shown) and analyzed as described in the legend to Fig. 4.

(e.g., see Fig. 6). Therefore, the extended runout periods in the presence of rifampin at this late time might reflect just slowed replication fork movement rather than resistance to rifampin.

DNA synthesis in *rnh* mutants during inhibition of protein synthesis by chloramphenicol treatment. The typical pattern of DNA accumulation in the presence of chloramphenicol in a *dnaA*$^+$ *rnh-224* strain is shown in Fig. 1B. The rate of DNA synthesis remained fairly constant for the first 60 min, and

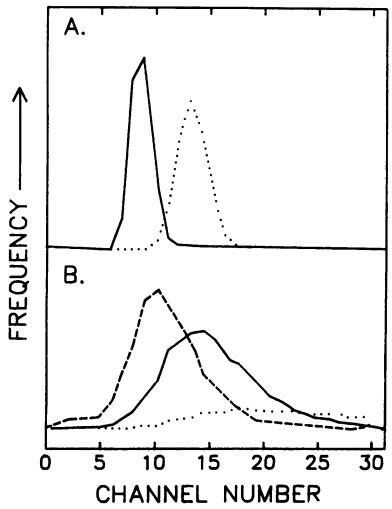

FIG. 4. Relative cell size distributions in populations of *rnh* mutant strains. Size distributions of one-, two-, and three-genome-equivalent cells in populations of CM3590 (*rnh*$^+$ *dnaA*$^+$) grown at 30°C (A) and CM3592 (*rnh-224 dnaA46*) at 41°C (B) were obtained by drawing out the cross-section of the histograms shown in Fig. 2F and H, respectively, at fluorescence (DNA) values corresponding to one (dashed lines), two (solid lines), and three (dotted lines) genome equivalents. The relative sizes are shown by the channel number of the light scatter signal.

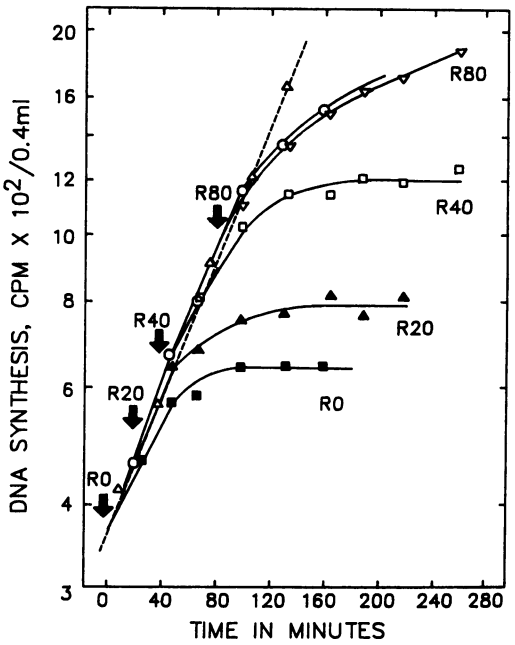

FIG. 6. DNA synthesis in the presence or absence of chloramphenicol and rifampin. A culture of CM3592 (*rnh-224 dnaA46*) grown exponentially at 41°C was labeled with [^{14}C]uracil (△). Chloramphenicol (150 μg/ml) was added to a portion (○) of the culture at time zero. At the times indicated by arrows (■, R_0; ▲, R_{20}; □, R_{40}; ▽, R_{80}), rifampin (at 200 μg/ml) was added to the chloramphenicol-treated culture. DNA synthesis in these subcultures was measured as described in the legend to Fig. 1. The amounts of residual DNA synthesis (*dG*; the net increase in counts per minute) in the presence of rifampin for R_0, R_{20}, R_{40}, and R_{80} were 270, 330, 580, and ca. 1,000 cpm, respectively. The relative values of residual DNA synthesis (*dG/G*; the *dG* value divided by the amount of radioactivity [*G*] at the addition of rifampin) were 0.75, 0.72, 0.94, and 1.04, respectively.

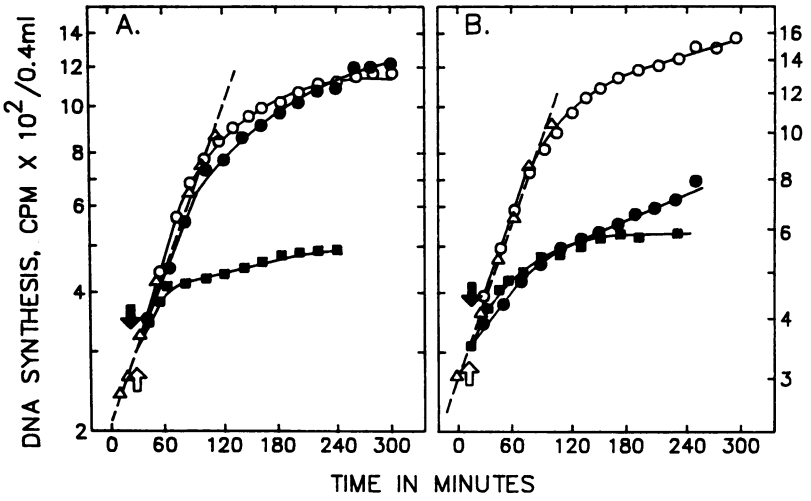

FIG. 7. Effects of the *relA1* mutation on DNA synthesis during tryptophan starvation. Cells of AQ3497 (*rnh-224 dnaA46 relA1*) (A) and AQ3405 (*rnh-224 dnaA47 relA*$^+$) (B) were grown exponentially and labeled with [^{14}C]uracil at 41°C (△). Chloramphenicol (○) and rifampin (■) were added to portions of the cultures at the times indicated by the open and closed arrows, respectively. A third portion of the cultures (●) was shifted to tryptophan-limiting medium to attain tryptophan starvation (see Materials and Methods). The onset of tryptophan starvation was at 26 min in panel A and at 24 min in panel B. DNA synthesis was measured as described in the legend to Fig. 1.

then it decreased to about half this rate, and later, at 200 min, to approximately one-third the initial rate, at which it appeared to continue for a long period (9). The picture is different for the *dnaA*::Tn*10 rnh-224* strain CM3847 (Fig. 1C) or the *dnaA*(Ts) *rnh-224* strain CM3592 at 41°C (Fig. 6). In these strains the rate of DNA synthesis (i.e., the slope of accumulation curves) continued to increase exponentially after chloramphenicol addition, reaching a 2- to 2.5-fold-higher value 60 to 80 min later (all at 41°C). After 80 to 100 min the rate decreased gradually, approaching a value roughly equal to the one at the moment of chloramphenicol addition. (Note: The data are presented in semi-logarithmic plots.)

Although there is this large difference between the *dnaA*$^+$ *rnh-224* cells (Fig. 1B) and *dnaA rnh* double mutant cells (Fig. 1C and Fig. 6), it turns out that the amount of cSDR in all cases was the same when calculating absolute rates of DNA synthesis and correcting for the contribution of the normal DNA replication to the overall runout of DNA synthesis in chloramphenicol. When subtracting the estimated runout of normal DNA synthesis (dotted line in Fig. 1B) from the actual DNA synthesis curve in chloramphenicol in strain CM3591 (Fig. 1B), exactly the same amounts of DNA per unit cell mass (A_{450}) were accumulated owing to cSDR in the *dnaA*$^+$ strain CM3591 (Fig. 1B) and the *dnaA* mutant strain CM3847 (Fig. 1C). The following example should clarify this point. At 160 min (i.e., approximately 120 min after chloramphenicol addition) 900 cpm had accumulated in strain CM3591 (difference between the actual curve and dotted normal runout curve in Fig. 1B). At the same time a total of 550 cpm had accumulated in strain CM3847 in the presence of chloramphenicol (Fig. 1C). Taking into account the difference in A_{450} at the moment of chloramphenicol addition (i.e., 0.36 in the former and 0.22 in the latter), we found that in both cases the accumulation during 120 min in chloramphenicol was 2,500 cpm per unit A_{450}. Furthermore, the rates of DNA accumulation per unit A_{450} were practically identical in the two strains later than 80 min after chloramphenicol addition.

We can therefore conclude that the cSDR in the presence of chloramphenicol is independent of the genetic constitution of the strains with respect to *dnaA*. The rate of cSDR in the presence of chloramphenicol seems to be independent of the concentration of chromosomal DNA as this is twofold lower in the *dnaA46 rnh-224* strains CM3592 (at 41°C) and CM3847 than in the *dnaA*$^+$ *rnh-224* strain CM3591 (Table 2). This suggests that, within this range, the concentration of *oriK* is not limiting the rate of cSDR. However, the conditions for initiating cSDR at *oriK* in the two situations are most likely not the same. Thus, the fact that the level of cSDR was the same in the two cases may be fortuitous. Whether cSDR also runs during exponential growth at the same rate in strain CM3591 (*dnaA*$^+$ *rnh*) as in the *dnaA rnh* double mutant, i.e., unaffected by the presence of the normal replication system, cannot be precisely judged. However, it is clear that cSDR is functioning under *dnaA*$^+$ *rnh* conditions on top of the normal replication. This is corroborated both by the finding that DNA content increased 10% in the *dnaA*$^+$ *rnh-224* strain as compared with the *dnaA*$^+$ *rnh*$^+$ strain (Table 2) and by the presence of cells with too much DNA in the CM3440 (*dnaA*$^+$ *rnh*) populations (Fig. 5). In fact, it had previously been demonstrated that *oriK*'s are being used in the *rnh-224 dnaA*$^+$ strain although *oriC* is the dominant origin of replication (7).

With respect to the DNA synthesis pattern in the presence of chloramphenicol, it is important to note that the rate of DNA synthesis increased in the *dnaA rnh* double mutant after addition of the inhibitor (Fig. 1C and 6). The DNA accumulation curves followed (Fig. 1C) or even slightly exceeded (Fig. 6) the one for the untreated controls. This means that initiation of cSDR occurred at an exponentially increasing rate after chloramphenicol addition. Thus, the pace of initiation of cSDR set during exponential growth was not disturbed by blockage of protein synthesis. This conclusion is supported by the measurements of residual DNA synthesis in the presence of rifampin. When inhibiting initiations of cSDR with rifampin at different times after chloramphenicol addition (Fig. 6), we found that the amount of residual DNA synthesis (*dG*; see the legend to Fig. 6) increased severalfold over a period of 80 min. The relative value of residual DNA synthesis (*dG*/*G*) increased from 0.75 (R_0) to 0.94 (R_{40}). This indicates that chloramphenicol treat-

ment actually resulted in a 25% stimulation of cSDR initiations during the first 40 min. Later, as DNA accumulation slowed down, the frequency of cSDR initiation seemed to decrease, most likely concomitantly with a decrease in the rate of replication fork movement.

cSDR during amino acid starvation. The stimulation of the initiation of cSDR by chloramphenicol addition beyond the normal pace was reminiscent of the stimulation of initiation of normal replication in *dnaA46* mutants (23). Since the latter stimulation was shown to be subject to stringent control (29), we analyzed cSDR during amino acid starvation in stringent (*relA*$^+$) and relaxed (*relA1*) derivatives of the *dnaA46 rnh-224* strain at 41°C (Fig. 7). While the cSDR continued during amino acid starvation of the *relA1* strain AQ3497 (Fig. 7A) at the same pace as during chloramphenicol treatment of either the *relA*$^+$ or *relA* strain (Fig. 6 and 7A and B), it was drastically inhibited after deprivation of the *relA*$^+$ strain AQ3405 for tryptophan (Fig. 7B). Using rifampin to probe for initiations occurring during amino acid starvation, we found that very few extra initiations occurred within the first 80 min of starvation (data not shown). Addition of tryptophan alone or together with chloramphenicol after 80 min of starvation of the *relA*$^+$ strain AQ3405 (Fig. 7B) resulted in a threefold stimulation of DNA synthesis within 30 min (data not shown). These treatments lead to a suppression of ppGpp formation (5). We therefore conclude that cSDR initiaions are subject to stringent control, i.e., are sensitive to the presence of ppGpp.

DISCUSSION

The experiments described in the present report have yielded the following information about the characteristics of the cSDR initiation process. (i) In the absence of the normal replication system, cSDR allows the cells to grow at a 30 to 40% reduced growth rate and with a twofold-decreased DNA content (Table 2). (ii) cSDR initiations appear to be random with respect to time in the cell cycle as well as choice of origins (Fig. 3). (iii) The frequency of cSDR initiation continues to increase exponentially for more than one doubling time after inhibition of protein synthesis with chloramphenicol (Fig. 1C, 6, and 7). (iv) cSDR initiation is inhibited during amino acid starvation in stringent (*relA*$^+$) but not in relaxed (*relA1*) strains, i.e., it is sensitive to ppGpp (Fig. 7). (v) cSDR initiation is rifampin sensitive, demonstrating an involvement of RNA polymerase (Fig. 6). (vi) cSDR functions in *dnaA*$^+$ *rnh-224* strains in parallel to the normal *oriC*$^+$ *dnaA*$^+$-dependent chromosome replication system (Fig. 1).

A most significant, although not totally unexpected, finding in this study is the random mode of cSDR initiation. cSDR appears to be initiated randomly both in time and in place, i.e., in terms of choice among the several different *oriK*'s. Consequently, cell mass and DNA distributions are very heterogeneous (Fig. 2G and H). In *dnaA*$^+$ *rnh*$^+$ cells initiation of normal replication at *oriC* correlates fairly well with the reaching of a certain cell mass per volume (8; for a review, see reference 37). Furthermore, the initiation of normal replication appears to take place almost simultaneously at independent *oriC* sites in the same cell (30). Previously, we have shown that a population of *rnh* mutant cells accumulated a large fraction of cells with three completed chromosomes after inhibition of initiation of cSDR by inactivation of the RecA protein (12). This finding supports the conclusion of randomness of cSDR initiations, since the appearance of cells with three or five chromosomes is a reflection of imprecise timing of initiations as discovered by Skarstad et al. (30) for *dnaA46* mutants.

Despite the complete randomness of cSDR initiations, viable and exponentially growing populations are obtained as is the case with cells in which the chromosome is driven by the plasmid R1 origin (16). For cSDR, however, it is just one step more random as initiations can occur at four different sites. Thus, the precise timing of the *dnaA*$^+$ *oriC*$^+$ initiation pathway (22, 30, 32) appears not to be essential for growth and cell division under certain growth conditions.

cSDR initiation at a single *oriK* can be concluded to occur at a rather low frequency only: the rate of total DNA synthesis is three- to fourfold lower in the *dnaA rnh* double mutants than in the wild type. Assuming that there are just four different *oriK*'s (7) and that these are used with an equal probability, we can conclude that the frequency (per unit mass) with which each individual *oriK* functions in initiation is 12 to 16 times lower than for *oriC* under the same growth conditions. Obviously, not all *oriK*'s are used in every mass doubling time. Otherwise DNA content should be increased and cell size distribution should be more homogeneous. Unlike the normal initiation of chromosomal replication (37), cSDR initiations thus are not well adjusted to the cell growth. We must conclude that cells in which DNA synthesis operates exclusively under the cSDR system are only viable because the multiple *oriK*'s together yield an overall initiation frequency which, although three- to fourfold lower than in *oriC*$^+$ *dnaA*$^+$ cells, allows the cells to reach a low but sufficient DNA/mass ratio, sufficient for reaching a 30 to 40% diminished growth rate relative to wild type. A cell with a single *oriK* could hardly be expected to grow.

cSDR initiation can now be envisaged to occur at the *oriK* sites by interaction of RecA protein with a transcript synthesized by RNA polymerase, leading to the formation of an RNA-DNA hybrid which persists owing to the absence of RNase H. The synthesis of the transcript is sensitive to ppGpp and can, probably as a consequence of this ppGpp sensitivity, be slightly stimulated by chloramphenicol treatment. Whether the transcript actually serves as a primer of DNA synthesis is not known. It is quite possible that the RNA-DNA hybrid and displaced single-strand DNA stabilized by RecA protein provide an initiation structure at *oriK* at which a replisome can set into action the DnaG primase actually priming DNA synthesis, as suggested in in vitro initiation at *oriC* (2, 35). The formation of such a structure appears to be an infrequent and stochastic event since the frequency of cSDR is only 0.1 to 0.2 per *oriK* per doubling time and virtually uncoupled from the cell mass.

The discovery of the sensitivity of *oriK* initiation to the stringent control raises the possibility that *rrn* operons encoding rRNAs are sites for the formation of such structures. Comparison of the map positions of the seven *rrn* operons (1) and the four *oriK* sites (7), however, indicates that they do not coincide. Although a hasty conclusion must be avoided because of the imprecision of the *oriK* mapping, it is clear that at least the two *oriK* sites mapping in the terminus region of chromosome replication (*terC*) cannot be *rrn* operons. Whatever the nature of the sites for *oriK* transcription is, the ppGpp sensitivity of cSDR suggests regulation of overall cSDR initiations coupled to ribosome (rRNA) biosynthesis.

Stable DNA replication (iSDR) can be induced in *rnh*$^+$ cells after SOS-inducing treatments (11, 14). However, no significant change in levels of RNase H activity during induction has been detected (3), suggesting that cSDR is not a constitutive expression of an inducible system (i.e., iSDR) by a mutation. Support for this inference comes from the differences in the characteristics of the two replication

systems (reviewed in reference 10). While iSDR may be a backup replication system alternative to the *oriC*$^+$ *dnaA*$^+$-dependent pathway (14, 19), it is possible that cSDR is a genetic artifact fortuitously manifested by RNase H deficiency, eliminating the specificity of the chromosomal replication machinery for *oriC* (13, 28). The cSDR initiation system may actually be a remnant of a primitive replication system which may turn out to be related to present-day initiation mechanisms of plasmids such as ColE1.

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