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RecA Protein Acts at the Initiation of Stable DNA Replication in *rnh* Mutants of *Escherichia coli* K-12

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Escherichia coli rnh **mutants lacking RNase H activity are capable of *recA*$^+$-dependent DNA replication in the absence of concomitant protein synthesis (stable DNA replication). In *rnh dnaA*::Tn*10* and *rnh* Δ*oriC* double mutants in which the *dnaA*$^+$-dependent initiation of DNA replication at *oriC* is completely blocked, the *recA200* mutation encoding a thermolabile RecA protein renders both colony formation and DNA synthesis of these mutants temperature sensitive. To determine which stage of DNA replication (initiation, elongation, or termination) was blocked, we analyzed populations of these mutant cells incubated at 30 or 42°C in the presence or absence of chloramphenicol (CM) by dual-parameter (DNA-light scatter) flow cytometry. Incubation at 30°C in the presence of CM resulted in cells with a continuum of DNA content up to seven or more chromosome equivalents per cell. The cultures which had been incubated at 42°C in the absence or presence of CM consisted of cells with integral numbers of chromosomes per cell. It is concluded that active RecA protein is required specifically for the initiation of stable DNA replication.**

Chromosome replication in *Escherichia coli* can be dissected into three stages: initiation, elongation, and termination. Each stage involves specific gene products, e.g., *dnaA* and *rpoB* in initiation; *dnaE*, *dnaG*, *dnaN*, *dnaX*, and *dnaZ* in elongation; and *dnaT* in termination (for a review, see reference 7). The products of some genes, such as *dnaB* and *dnaC*, are required for both the initiation and elongation stages. Whereas inactivation of the product of a gene involved in the elongation stage results in the immediate cessation of DNA replication, loss of an initiation gene activity prevents the initiation of a new round at the unique origin of replication, *oriC*, but allows the completion of the round already underway. Thus, *dnaA*(Ts) mutants can carry out chromosome replication to the terminus (*terC*) at the restrictive temperature, and the two chromosomes are segregated into daughter cells on cell division (15).

Both in vivo and in vitro experiments have indicated that RNase H encoded by the *rnh*$^+$ gene is required for the initiation of a reproductive DNA replication cycle to occur exclusively at *oriC* (2, 6, 8). In *rnh* mutants (formerly *sdrA* [12]), replication begins from several sites other than *oriC* in a *dnaA*$^+$-independent fashion (2). Owing to this secondary replication system activated by *rnh* mutations, *rnh* mutants can dispense with the *oriC* site and the *dnaA* gene (6). DNA replication in the absence of RNase H can continue under conditions that inhibit protein synthesis, e.g., in chloramphenicol (CM), in contrast to normal initiation at *oriC*, which requires continued protein synthesis. This replication in *rnh* mutants in the absence of protein synthesis has been termed constitutive stable DNA replication (cSDR) (4). Induced stable DNA replication (iSDR) can be elicited in wild-type (*rnh*$^+$) cells by treatments inducing the SOS response (5).

A unique property of both cSDR and iSDR is the requirement for the *recA*$^+$ gene product. For example, cSDR in *rnh* mutants cannot take place when RecA protein is inactivated (13). In *rnh dnaA*::Tn*10* and *rnh* Δ*oriC* mutants in which *dnaA*$^+$ *oriC*$^+$-dependent initiation is completely blocked (6), introduction of the *recA200* mutant allele encoding a temperature-sensitive RecA protein renders both colony formation and DNA replication of these mutants temperature sensitive (T. Kogoma, N. L. Subia, and K. von Meyenburg, submitted for publication). In this study, we used dual-parameter flow cytometry techniques (1, 9) to determine which stage of DNA replication is blocked in these mutants by the thermal inactivation of RecA200 protein. The results indicate an involvement of RecA protein in the initiation step of cSDR.

MATERIALS AND METHODS

E. coli **strains and culture conditions.** The construction of strains AQ2096 (*rnh-224 dnaA850*::Tn*10*), AQ2118 (*rnh-224* dnaA850::Tn*10 recA200*), and AQ2120 (*rnh-224* Δ*oriC1071 dnaA5 recA200*) has been described elsewhere (Kogoma et al., submitted). PC5 (*dnaA5*) is a temperature-sensitive DNA initiation mutant, and PC3 (*dnaG3*) is a temperature-sensitive DNA elongation mutant (15). Cultures were grown at 30°C in L broth or M9 glucose supplemented with Casamino Acids (0.2%; Difco Laboratories, Detroit, Mich.), other amino acids (50 μg/ml), thymine (8 μg/ml), and thiamine hydrochloride (2 μg/ml) as required (4). [*methyl*-^{3}H]thymine (5 mCi/0.036 mg) and [2-^{14}C]thymine (0.1 mCi/0.27 mg) were obtained from New England Nuclear Corp., Boston, Mass.

Measurement of DNA synthesis. Cultures were labeled with [^{3}H]thymine (10 μCi/8 μg per ml), 0.1-ml samples were withdrawn at intervals, and radioactivity in acid-insoluble material was determined as previously described (4).

Fixation and staining. Cells were fixed with ice-cold 70% aqueous ethanol and stored in the cold. For staining, fixed cells were suspended in 0.01 M Tris (pH 7.4)–0.01 M $MgCl_2$ containing the DNA-specific fluorescent dye, 90 μg of plicamycin (Pfizer Inc., New York, N.Y.) per ml, and 20 μg of ethidium bromide (Calbiochem-Behring, La Jolla, Calif.) per ml.

Flow cytometry. Measurements were carried out with a

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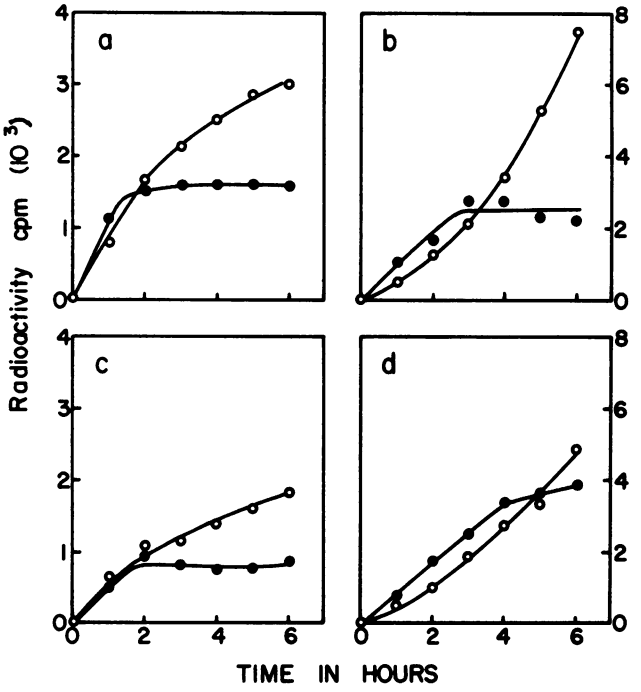


FIG. 1. DNA synthesis in strains AQ2118 and AQ2120 at 30 or 42°C in the presence or absence of CM. Cultures of strains AQ2118 (*rnh-224 dnaA*::Tn*10 oriC*$^+$ *recA200*) (a and b) and AQ2120 (*rnh-224 dnaA5* Δ*oriC1071 recA200*) (c and d) were grown at 30°C to titers of 2.1×10^8 and 1.1×10^8 cells per ml, respectively. The doubling times of AQ2118 and AQ2120 were 130 and 170 min, respectively. [^{3}H]thymine (10 μCi/8 μg per ml) was added to portions of the cultures, which then were incubated in the presence (a and c) or absence (b and d) of CM (150 μg/ml) at 30 (○) or 42°C (●). Samples were withdrawn at intervals, and radioactivity in acid-insoluble material was determined (4).

laboratory-built, microscope-based flow cytometer previously described in detail (10). The intensity of fluorescent light (in proportion to cellular DNA content) and the amount of scattered excitation light (in proportion to protein content) (1) for single cells were simultaneously measured at rates of 10^3 to 10^4 cells per s. The data were stored and analyzed by a multichannel pulse-height analyzer as previously described (1, 9). Calibration of the fluorescence axis to cellular DNA content was based on an analysis of a *dnaA*(Ts) mutant culture (see Fig. 3b) which had been grown for 90 min at 42°C as previously described (11).

RESULTS

DNA replication in the absence of *dnaA*$^+$ *oriC*$^+$-dependent initiation. *rnh dnaA*::Tn*10 recA200* (*oriC*$^+$) and *rnh* Δ*oriC1071 recA200* (*dnaA5*) mutants ceased DNA synthesis within 2 h on incubation at 42°C in the presence of CM (Fig. 1a and c). Temperature shift had little effect when the *recA*$^+$ allele replaced the *recA200* mutation in these strains (Kogoma et al., submitted) or when the *rnh dnaA*::Tn*10 recA200* strain harbored a plasmid (pBEU34) (14) which carried the *recA*$^+$ gene (data not shown). Therefore, the temperature sensitivity of cSDR in these mutants was due to the *recA200* allele present.

DNA replication in *rnh dnaA*::Tn*10 recA200* mutants was temperature sensitive even in the absence of CM (Fig. 1b). The cessation of DNA synthesis at 42°C was, however, slower in the absence of CM (Fig. 1b) than in its presence (Fig. 1a). This may be imputed to a delay in the actual decrease in RecA activity at 42°C owing to the continued synthesis of the mutant RecA protein.

A considerable degree of DNA synthesis also occurred in the *rnh* Δ*oriC recA200* (*dnaA5*) mutant at 42°C when protein synthesis was allowed (Fig. 1d). The reason for this delay is not understood. We considered the possibility that DnaA protein was involved in DNA synthesis in the absence of both the *oriC* site and RecA activity and that the delay was a result of slow inactivation of the DnaA5 mutant protein under this condition. This possibility was ruled out because the introduction of a high-copy-number plasmid carrying the *dnaA*$^+$ gene (resulting in about a sixfold overproduction of DnaA protein) into this mutant did not enhance DNA replication at 42°C (data not shown). Whatever the cause for the delay was, however, the continuing DNA synthesis was not sufficient for cell survival, since the mutant could not form colonies at 42°C.

In summary, these results indicate that in the *rnh* mutants that lack the *dnaA*$^+$ *oriC*$^+$-dependent initiation mechanism, active RecA protein becomes essential for DNA replication during the cell cycle and thus for cell viability.

Some degradation of DNA has been shown to occur in *recA* mutants with certain genetic backgrounds (16). When the stability of prelabeled DNA was examined under conditions similar to those of the experiments above, 15 to 20% of the labeled DNA became acid soluble during 6 h of incubation at 42°C in the presence of CM, whereas no DNA breakdown was detected during similar incubation at 30°C (Fig. 2a). There was no appreciable DNA degradation in the absence of CM at either temperature (Fig. 2b).

Flow cytometric analysis of cell populations arrested at an initiation or elongation step. PC5 is a *dnaA*(Ts) mutant strain which, at the restrictive temperature, is able to complete rounds of DNA replication and to undergo subsequent cell division but is unable to initiate new rounds of replication (15). The flow cytometric analysis of PC5 cultures grown at 30 and 42°C (Fig. 3a and b) indicated that the culture grown at 30°C consisted of cells with a continuum of DNA content ranging from two to seven chromosome equivalents, and the culture grown for 90 min at 42°C contained only cells with

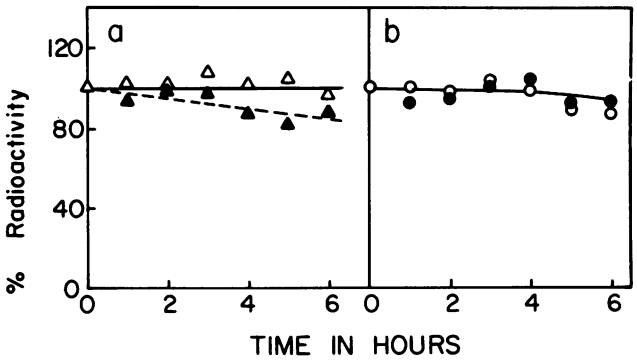


FIG. 2. Stability of prelabeled DNA in strain AQ2118 during incubation in the presence or absence of CM. An exponential culture of strain AQ2118 was labeled with [^{14}C]thymine (4 μCi/8 μg per ml) for about two doublings at 30°C. Cells were then washed by filtration and suspended in warm, nonradioactive medium. Samples were incubated at 30 (△,○) and 42°C (▲,●) with (a) or without (b) CM (150 μg/ml). Samples were withdrawn, and radioactivity in acid-insoluble material was determined. Radioactivities of the zero time samples (100%) were 231 and 249 cpm for panels a and b, respectively.

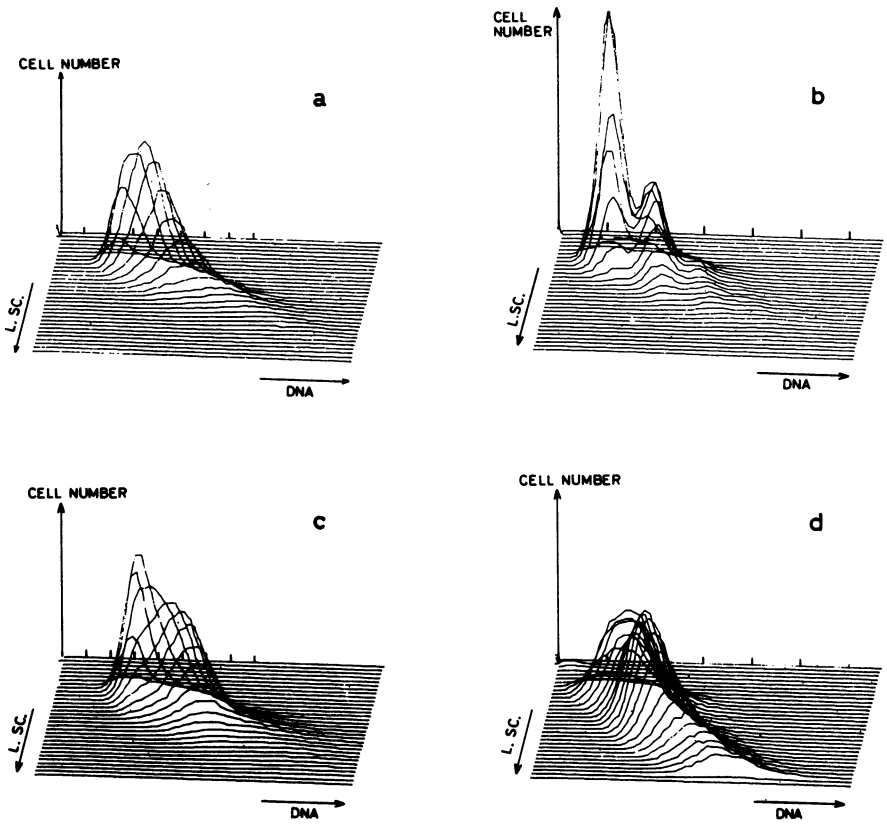


FIG. 3. Three-dimensional DNA-light scatter histograms of PC5 and PC3 cultures grown at 30 or 42°C. PC5 (a and b) and PC3 (c and d) cultures were grown in L broth at 30°C to an early log phase (ca. 10^8 cells per ml). The doubling times of PC5 and PC3 were 33 and 50 min, respectively. The cultures were divided into two portions: one (a and c) incubated at 30°C for 60 min and the other (b and d) incubated at 42°C for 90 min. Cells were then fixed with ethanol and analyzed by dual-parameter flow cytometry as described in the text. Three-dimensional histograms accumulated in a multichannel (63 by 63) pulse-height analyzer are shown with the cell number along the *z* axis, the fluorescent intensity (DNA) along the *x* axis, and the light scattered by each cell (L. SC.) along the *y* axis. The units on the *x* (DNA) axis represent integer numbers of fully replicated chromosomes. The amount of fluorescent light from a fully replicated chromosome of *E. coli* was determined by the positions of peaks in the histogram (b) of the *dnaA*(Ts) mutant at 42°C as previously described (11).

discrete amounts of DNA, i.e., one (1n) and two (2n) chromosome equivalents. On the other hand, a similar incubation of PC3, a *dnaG*(Ts) strain, at 42°C resulted in histograms with a continuous distribution of DNA content (Fig. 3d). This was expected from the immediate arrest of DNA replication in the elongation mutant at the restrictive temperature owing to the inactivation of the thermolabile primase, the *dnaG*(Ts) gene product. A comparison of the histograms for cultures grown at 30 and 42°C (Fig. 3c and d) also revealed filamentation of the mutant cells at 42°C, a characteristic of DNA synthesis arrest in an elongation mutant (15).

The average DNA contents of the 42°C cultures of the *dnaA5* and *dnaG3* mutants (Fig. 3b and d, respectively) were significantly less than those of the respective 30°C cultures (Fig. 3a and c). This can be accounted for by the limited but significant degrees of cell division that occurred after temperature shift. The cell numbers of the *dnaA5* and *dnaG3* cultures increased by 4.7- and 2.2-fold, respectively, during the 90-min incubation period at 42°C. In general, completion of a round of chromosome replication is a prerequisite for cell division (3), and elongation mutants such as *dnaG*(Ts) strains therefore stop dividing at the restrictive temperature. The observed residual cell division of the *dnaG3* culture at 42°C was presumably performed by cells that had entered the D period (the time interval between the termination of chromosome replication and subsequent cell division) before temperature shift (Fig. 3c).

These results demonstrate that flow cytometry provides a means to distinguish between two cell populations arrested at the initiation and elongation stages of DNA replication.

Effects of inactivation of RecA protein in the presence of CM. If functional RecA protein is required specifically at a step in the initiation of stable DNA replication (SDR), inactivation of RecA protein should allow completion of the round of DNA replication already underway without the initiation of new rounds of replication. Such a population would consist of cells with discrete amounts of DNA per cell. On the other hand, if active RecA is needed for DNA chain elongation, the loss of RecA activity would result in a cell population consisting of cells with a continuum of DNA content.

The three-dimensional histograms shown in Fig. 4 and 5 are a summary of the flow cytometric analyses of the *rnh* mutant cultures treated under various conditions. The kinetics of DNA synthesis in the same cultures under these conditions are presented in Fig. 1 as described above. A culture of the *rnh-224 dnaA*::Tn*10 recA200* mutant growing exponentially at 30°C consisted mainly of cells containing amounts of DNA between 1n and 3n (Fig. 4a). After a 4-h

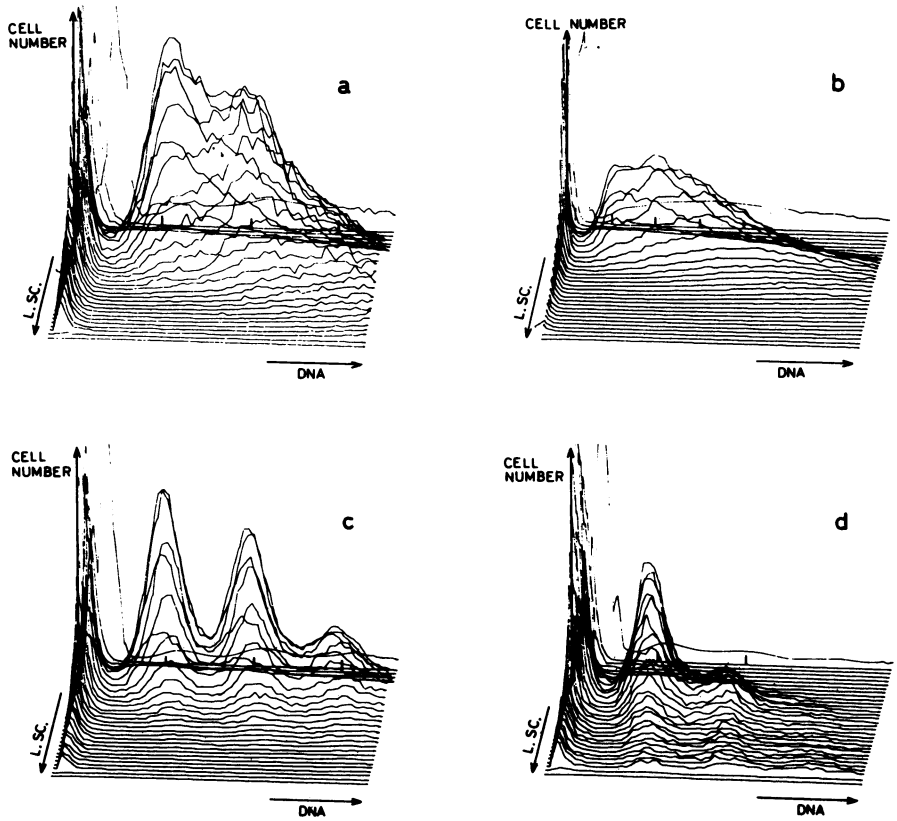


FIG. 4. Three-dimensional histograms of strain AQ2118 cultures grown at 30 or 42°C in the presence or absence of CM. A culture of strain AQ2118 was grown to a mid-log phase (2.1×10^8 cells per ml) at 30°C (a) as described in the legend to Fig. 1. Portions of the culture were further incubated in the presence of CM (150 μg/ml) at 30 (b) or 42°C (c) for 4 h or in the absence of CM at 42°C for 6 h (d). The fluorescence signals (noise) at or near zero DNA content were presumably due to the presence of cell debris in the cultures. The possibility that these cultures contained DNA-less cells was not examined. For axis designations, see the legend to Fig. 3.

incubation period at 30°C in the presence of CM, the proportion of cells with more than 3n equivalents of DNA drastically increased (Fig. 4b). This increase in the DNA content was not accompanied by a significant increase in the average cell mass. This was expected from the continuous DNA replication in these *rnh* mutants in the absence of protein synthesis (4). In contrast to the broad and continuous distribution of the DNA content of the 30°C cells, a 4-h incubation period at 42°C resulted in cells containing discrete amounts of DNA (Fig. 4c). Very similar results were obtained with *rnh-224 ΔoriC1071 recA200* mutants (Fig. 5a and c).

It should be noted that these cell populations contained 3n cells, suggesting the occurrence of asymmetric initiation of replication in *rnh* mutants. For example, premature initiation might have occurred at only one of a pair of origins located on the two partially replicated regions of a replicating chromosome; on completion of the rounds of replication, such a molecule would be resolved into three fully replicated chromosomes.

Cells of a *recA*$^+$ *rnh-224 dnaA*::Tn*10* strain which were treated similarly at 42°C contained amounts of DNA which were indiscrete (Fig. 6). A similar result was obtained with a *recA*$^+$ *rnh-224 ΔoriC1071* strain (data not shown). Thus, the accumulation of cells with integral numbers of chromosomes was a consequence of the loss of a functional RecA protein.

Effects of inactivation of RecA protein in the absence of CM. The result of an analysis of an *rnh-224 dnaA*::Tn*10 recA200* mutant culture that was incubated for 6 h at 42°C in the absence of CM is illustrated in Fig. 4d. A majority of the cells in this population had one fully replicated chromosome, and a small fraction contained two chromosomes. A comparison of this population with the exponentially growing cells at 30°C (Fig. 4a) revealed a significant decrease in the number of 2n cells during the incubation at 42°C, indicating that cell division was continuing. The effect of incubation at 42°C in the absence of CM was only marginal for the Δ*oriC* counterpart (Fig. 5d). This could be attributed to the long lag in the inhibition of DNA synthesis under these conditions, as described above (Fig. 1d).

DISCUSSION

rnh recA(Ts) double mutants have been shown to cease DNA synthesis at 42°C with a total increase of 40 to 45% of the amount present at the time of the addition of CM, whereas there was no inhibition of DNA replication in CM at 30°C (13). This suggested a possible involvement of a *recA* activity in the initiation of SDR. However, those mutants still had DNA replication initiated by the *dnaA*$^+$ *oriC*$^+$-dependent mechanism as well as SDR, complicating the interpretation of the results. In this study, we used *rnh dnaA*::*Tn10 recA*(Ts) and *rnh ΔoriC recA*(Ts) triple mutants in which the *dnaA*$^+$ *oriC*$^+$-dependent initiation pathway is completely blocked. The fact that both DNA synthesis (Fig. 1) and cell viability (Kogoma et al., submitted) are dependent on active RecA protein indicates that SDR becomes the reproductive DNA replication in these triple mutants. These mutant cells, after a period of incubation in the presence of

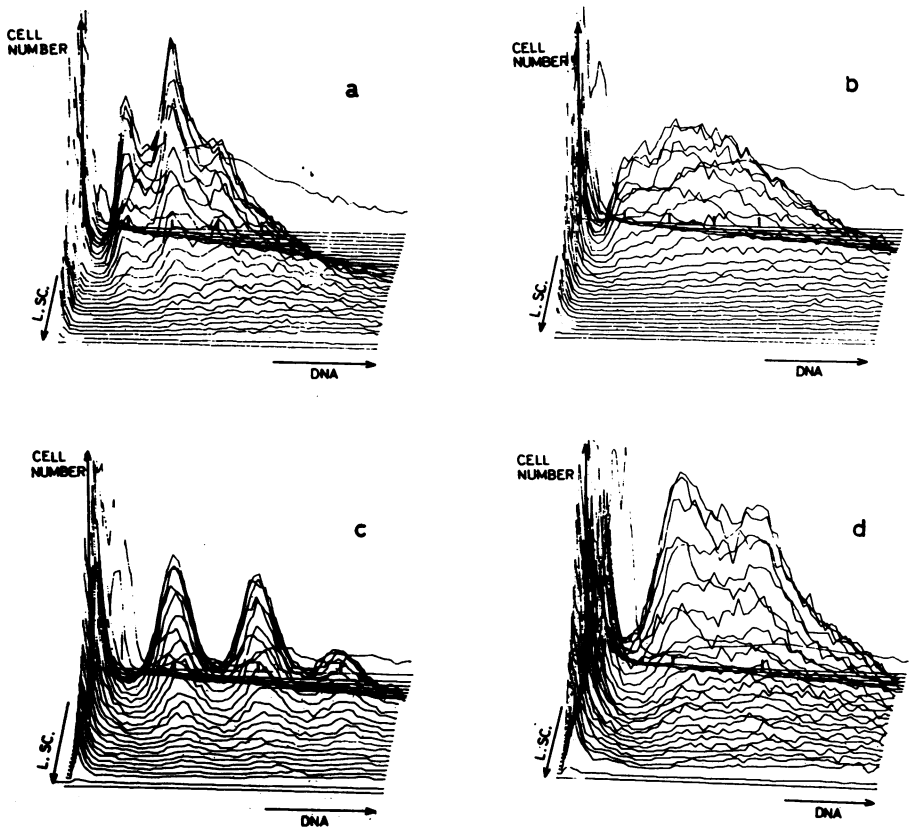
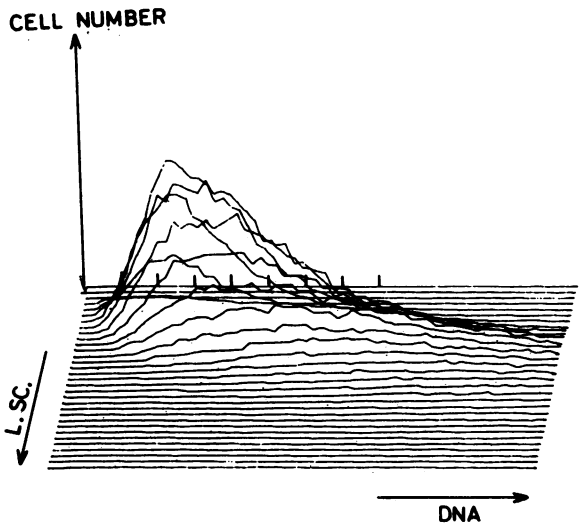


FIG. 5. Three-dimensional histograms of strain AQ2120 cultures grown at 30 or 42°C in the presence or absence of CM. A culture of strain AQ2120 was grown to a mid-log phase (1.1×10^8 cells per ml) at 30°C (a) as described in the legend to Fig. 1. Portions of the culture were incubated in the presence of CM at 30 (b) or 42°C (c) for 4 h or in the absence of CM at 42°C for 6 h (d). For axis designations, see the legend to Fig. 3.

CM at 42°C, contained integral numbers of chromosome equivalents (Fig. 4c and 5c), whereas their *recA*$^+$ counterparts did not (Fig. 6). This quantization of DNA content can best be explained as resulting from inhibition of initiation accompanied by successful completion of ongoing rounds of replication when the *recA*(Ts) protein is inactivated. Thus, RecA protein was required in a step in the initiation of SDR.



A moderate degree of breakdown of DNA during incubation at 42°C was detected (Fig. 2). However, breakdown of DNA is unlikely to cause the quantization of DNA content for several reasons. First, the amount of DNA lost by breakdown cannot account for the magnitude of the population change that occurred during incubation (e.g., from Fig. 4a to 4c). Second, if DNA quantization had been due to breakdown, it would have required a preferential loss of particular parts of the chromosome, e.g., only one of the two arms of each partially duplicated daughter chromosome, which is not a likely event. Additional evidence against this possibility comes from the observation that similar quantization occurred during incubation at 42°C in the absence of CM (Fig. 4d), in which little DNA breakdown was detected (Fig. 2).

It was conceivable that active RecA protein was required for the resolution of replicated daughter chromosomes at the end of a replication cycle and that loss of the *recA* function could therefore have resulted in a failure in segregation and reinitiation if resolution was prerequisite for the initiation process. Our results rule out this possibility. In the absence

FIG. 6. Three-dimensional histogram of strain AQ2096 grown at 42°C in the presence of CM. Strain AQ2096 (*rnh-224 dnaA*::Tn*10* *oriC*$^+$ *recA*$^+$) was grown at 30°C to a titer of 10^8 cells per ml and then incubated for 4 h at 42°C in the presence of CM. For axis designations, see the legend to Fig. 3.

of RecA function, chromosomes segregated into daughter cells when cell division was allowed in the absence of CM (Fig. 4d). Thus, we conclude that RecA protein is involved specifically in the initiation process of SDR.

What role does RecA protein play in the initiation of cSDR? It is not the activity of the protein to promote the cleavage of LexA repressor (13), nor does recombination per se appear to be involved in cSDR (N. L. Subia and T. Kogoma, manuscript in preparation). Consistent with these observations, extragenic suppressor mutations (*rin*) have been isolated which specifically suppress the adverse effects of several *recA* mutations (including deletion) on cSDR without alleviating the deficiency of the *recA* mutations in the recombination or LexA repressor cleavage functions (13). In the absence of *oriC*, *rnh* mutants initiate rounds of DNA replication from several sites (*oriK*) on the chromosome (2). The initiation of cSDR requires transcription (4; K. von Meyenburg and T. Kogoma, unpublished data). It is likely that transcripts, when allowed to form DNA-RNA hybrids in an extensive region in the absence of RNase H activity, can serve as primers for initiation at the *oriK* sites. RecA protein may be used to stabilize the displaced single-stranded DNA to facilitate interactions of the necessary components at the site (T. Kogoma, H. Bialy, N. L. Subia, T. A. Torrey, G. G. Pickett, and K. von Meyenberg *in* M. Schaechter, F. C. Neidhardt, J. Ingram, and N. O. Kjeldgaard, ed., *Molecular Biology of Bacterial Growth*, in press; Kogoma et al., submitted). The elucidation of the mechanistic role of RecA protein in this unique process awaits the cloning of *oriK* and subsequent studies of its interaction with RecA protein coupled with a thorough characterization of the *rin* mutations.

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