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Perturbed Chromosomal Replication in *recA* Mutants of *Escherichia coli*

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When initiation of DNA replication is inhibited in wild-type *Escherichia coli* cells by rifampin or chloramphenicol, completion of ongoing rounds of replication (runout of replication) leads to cells containing two, four, or eight fully replicated chromosomes, as measured by flow cytometry. In recombination-deficient *recA* strains, a high frequency of cells with three, five, six, or seven fully replicated chromosomes was observed in addition to cells with two, four, or eight chromosomes. *recA* mutants affected only in the protease-stimulating function behaved like wild-type cells. Thus, in the absence of the recombinase function of RecA protein, the frequency of productive initiations was significantly reduced compared with that in its presence. DNA degradation during runout of replication in the presence of rifampin was about 15%. The DNA degradation necessary to account for the whole effect described above was in this range or even lower. However, a model involving selective and complete degradation of partially replicated chromosomes is considered unlikely. It is suggested that the lack of RecA protein causes initiations or newly formed replication forks to stall but remain reactivatable for a period of time by functional RecA protein.

Initiation of DNA replication in *Escherichia coli* is a complex process in which several proteins act in succession to produce correctly positioned primed templates for bidirectional elongation (13). The DnaA protein is instrumental in this process, and we have shown that it is necessary for coordinating initiations from several origins within the same cell (24, 26). In addition, indications have been found that the RecA protein is also involved in initiation of chromosomal replication (24). It was demonstrated that *recA* mutant cells had the "asynchrony" phenotype. The method used to determine this phenotype was designed to measure the coordination of initiation at several origins within single cells and is described as follows. Initiation of replication, which requires a transcriptional event (14, 19) and de novo protein synthesis (15, 18), was inhibited with either rifampin (RIF, an inhibitor of transcription) or chloramphenicol (CAP, an inhibitor of protein synthesis) while ongoing rounds of replication were allowed to run to completion. Then the number of fully replicated chromosomes in each individual cell was measured by flow cytometry. In cultures of wild-type cells growing rapidly (doubling time, <60 min), almost all cells treated with RIF or CAP contained 2^n (n = 1, 2, or 3) chromosomes, showing that initiation occurred essentially simultaneously at the two, four, or eight origins within each cell, respectively. After drug treatment of a *recA56* mutant, a high frequency of cells with irregular numbers of chromosomes (different from 2^n, e.g., 3, 5, 6, or 7; the asynchrony phenotype) was found, suggesting that lack of a functional RecA protein caused perturbed initiation of replication.

The RecA protein of *E. coli* has several important functions. In the presence of ATP and single-stranded DNA, the protein is activated and stimulates self-cleavage of the LexA protein and phage repressors (16, 27, 28). The LexA protein represses a set of genes affecting cell division, mutagenesis, and SOS repair, including the *recA* gene (35). The RecA protein is also a DNA-dependent ATPase which catalyzes renaturation of complementary single-stranded DNA molecules, assimilates single strands into double-stranded molecules in a heteroduplex joint (20), and possesses a strand-separating activity on short duplex DNA molecules (2). Constitutive stable DNA replication, which is an alternative replication pathway occurring in *rnh* cells, is dependent on the RecA protein at the initiation stage (10, 11, 31).

In the present work, we studied the effect of different *recA* alleles on the asynchrony phenotype. We investigated whether the high level of DNA degradation observed in *recA* strains (33, 34) was responsible for the irregular numbers of chromosomes observed after RIF or CAP treatment. Based on our findings, different models for the possible function of the RecA protein are proposed and discussed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *E. coli* strains used (Table 1) were grown in Luria broth supplemented with 0.2% glucose and 10 mM phosphate buffer, pH 7.0. The temperature was 37°C unless otherwise stated. Culture growth rates were obtained by measuring the optical density at 600 nm.

Drug treatment. At an optical density of 0.1 to 0.2, RIF (150 μg/ml) or CAP (200 μg/ml) was added to inhibit initiation of replication. Incubation was continued for 3 to 4 (RIF) or 6 to 8 (CAP) generation times to let ongoing rounds of replication finish. To inhibit cell division during runout of replication, furazlocillin (FUZ, a kind gift from R. D'Ari) was added at a final concentration of 4 μg/ml. This drug binds to penicillin-binding protein 3, thereby selectively inhibiting septum formation (22). No cell lysis could be detected by using FUZ at the above concentration.

Fixation and staining. Cells were fixed in ice-cold 70% ethanol, washed in 10 mM Tris, pH 7.4, containing 10 mM $MgCl_2$, and stained in the same buffer with mithramycin (90 μg/ml) and ethidium bromide (20 μg/ml) as described previously (23, 24).

Flow cytometry. Flow cytometry was performed with a

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TABLE 1. *E. coli* strains

Strain	Relevant genotype	Source or reference
JC2924	*recA56*	A. J. Clark
JC2921	*recA1*	A. J. Clark
JC11266	*recA123 recA441*	A. J. Clark
JC9924	*recA430*	A. J. Clark
JC4728	*recA142*	A. J. Clark
N1331	*recA*$^+$	E. Seeberg (17)
N1332	*recA200*	E. Seeberg (17)

laboratory-built, microscope-based instrument (25, 29, 30). In short, cells were flowing one by one through a beam of excitation light, where each cell gave rise to a pulse of fluorescent light, the size of which was proportional to the amount of DNA-specific stain (mithramycin) bound. The excitation wavelength was selected so that ethidium bromide was not directly excited. Therefore, RNA-bound ethidium bromide did not contribute to the fluorescence signal. DNA-bound ethidium bromide was excited indirectly via DNA-bound mithramycin and was present to amplify the fluorescence signal. Fluorescence from and light scattered by each cell were quantitated simultaneously. The amount of light scattered by each cell has been found to be a good relative measure of cell mass (4; E. Boye, K. Skarstad, J. Valkenburg, and N. Nanninga, unpublished). Accumulation of the data in a multichannel analyzer resulted in a three-dimensional histogram with DNA content and scattered light as the parameters.

Average DNA content and DNA concentration. The dual-parameter histograms were integrated with respect to light scatter to yield single-parameter DNA histograms or with respect to DNA to yield single-parameter light scatter histograms. From these single-parameter distributions, average DNA content and cell mass were found. DNA concentration is the ratio of average DNA content to average cell mass.

DNA degradation. The amount of DNA breakdown during drug treatment was measured by labeling the DNA with [^{3}H]thymidine (Amersham, Buckinghamshire, England) for two generations, washing the cells in warm phosphate-buffered saline, pH 7.0, and reincubating in warm medium supplemented with unlabeled thymidine (50 μg/ml) for two generations. RIF and FUZ were added, and samples were withdrawn after 0, 30, 60, and 120 min into 5% trichloroacetic acid, filtered through glass fiber filters, washed with 10 mM HCl–96% ethanol, and dried, and the ^{3}H activity was measured by scintillation counting.

RESULTS

Synchrony of initiation in *recA* mutant cells. It has previously been shown that after RIF treatment, a culture of *recA56* cells contained a high frequency of cells with irregular numbers of chromosomes (24). Treatment with RIF allowed a large fraction of cells to divide, so that many cells otherwise destined to end up with five, six, seven, or eight chromosomes divided into cells with two, three, or four chromosomes. To be able to study DNA replication more specifically, FUZ was added to inhibit cell division in the present experiments. In strain JC2921 (*recA1*), inhibition by FUZ occurred rapidly, and less than 5% of the cells divided after RIF-FUZ treatment (Fig. 1). The three-dimensional DNA-light scatter histogram displayed multiple peaks representing cells with DNA contents corresponding to four, five, six, seven, or eight chromosome equivalents (Fig. 2B). Some cells (29% of the population) with large amounts of DNA (9 to 16 chromosome equivalents) were also found. There were more five-, six-, and seven-chromosome cells (45%) than four- and eight-chromosome cells together (25%; Tables 2 and 3). Thus, the *recA1* mutant clearly showed the asynchrony phenotype, like the *recA56* mutant reported previously (24).

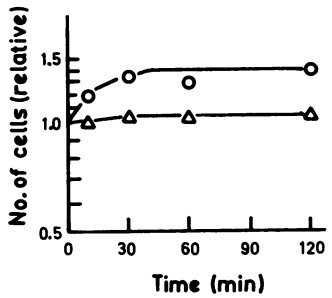

FIG. 1. Relative cell number in a culture of strain JC2921. The culture was split at the time of addition of either RIF (○) or RIF plus FUZ (△), and cell numbers were measured by quantitating and counting light scatter pulses by flow cytometry and normalizing the value at the time of drug addition to 1.0.

RIF treatment of different *recA* mutants. To find out whether this was an effect on *recA* strains in general, several different *recA* alleles were investigated for the asynchrony phenotype. The mutants tested were adversely affected in either all RecA protein activities, mainly the protease (regulatory) activity, or mainly the recombinase and DNA-binding activities.

Strains JC11266 (*recA123*) and JC2924 (*recA56*) have totally defective RecA proteins, and RIF or CAP treatment

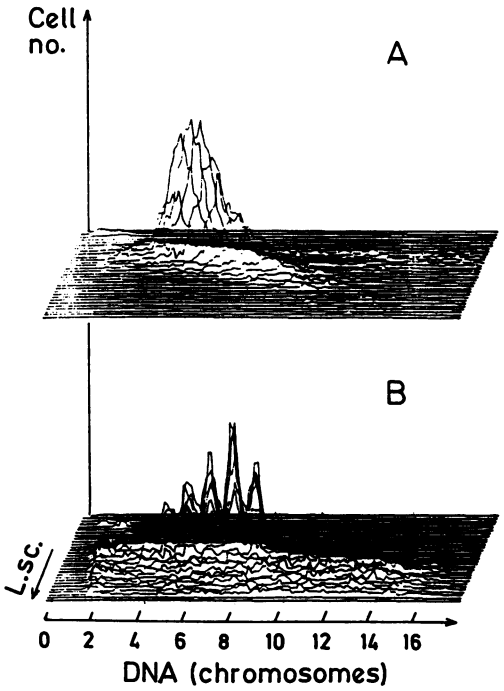

FIG. 2. DNA-light scatter (L.sc.) histograms of strain JC2921 growing exponentially at 37°C (A) or after treatment with RIF plus FUZ (B).

TABLE 2. Distribution of number of fully replicated chromosomes per cell in RIF-treated cultures[a]

Strain	*recA* allele	No. of chromosomes (% of cells)								
		1	2	3	4	5	6	7	8	>8
JC2924	*recA56*	8	9	10	15	12	10	9	7	
JC2921[b]	*recA1*	—	9[c]	—	8	10	13	17	13	29
JC11266	*recA123*	11	8	12	15	11	11	11	8	
JC9924	*recA430*	0	0	35[d]	18	0	0	15[e]	55	
JC4728	*recA142*	—	9[c]	8	16	14	15	15	11	
N1332[b,f]	*recA200*	2	13	22	25	11	9	6	3	
N1331[b,f]	*recA*$^+$	0	1	1	59	5	5	3	18	

[a] Treatment with CAP gave essentially the same results.
[b] Also treated with FUZ.
[c] Peaks were not separated; —, values pooled in neighboring column.
[d] Shoulder on peak stretching from 3.5 to 4 chromosome equivalents.
[e] Shoulder on peak stretching from 6 to 8 chromosome equivalents.
[f] Grown at 42°C.

yielded a high frequency of cells with irregular numbers of chromosomes (Table 2).

Strain JC9924 (*recA430*) is deficient in some of the RecA protease-stimulating functions and does not express UV mutability, but it is recombination proficient (3, 7). After RIF or CAP treatment, most of these cells contained four or eight chromosomes (Table 2). Both histogram peaks had shoulders toward lower DNA values (data not shown), suggesting either unfinished rounds of replication or some nonspecific DNA degradation during drug treatment.

Strain JC4728 (*recA142*), which is recombination deficient but has the protease-stimulating functions partly intact (5, 21), exhibited the asynchrony phenotype after drug treatment (Table 2). These data show that it is not the protease-stimulating function but rather the recombinase and DNA-binding activities of the RecA protein that are responsible for the asynchrony phenotype.

Synchrony and physiology of a *recA*(Ts) mutant. Strains N1331 and N1332 (*recA200*) were treated with RIF and FUZ at 42°C. The RecA200 protein is temperature sensitive and inactive at 42°C (17), while the otherwise isogenic counterpart N1331 is wild type at all temperatures. Most of the wild-type cells ended up with four or eight chromosomes (Fig. 3B and Table 2), but a major fraction of the N1332 cells ended up with three, five, six, and seven chromosomes (Fig. 3D; Tables 2 and 3). Strain N1332 thus showed the asynchrony phenotype at the restrictive temperature.

Indications of aberrant DNA replication were also seen in histograms of the exponentially growing cultures. The DNA-light scatter histogram of N1331 cells showed a narrow ridge stretching from low to high DNA and light scatter values, reflecting a tight correlation between DNA content and cell mass (Fig. 3A). In contrast, the histogram of exponentially growing N1332 cells exhibited a much broader ridge and an increased frequency of cells on both sides of the ridge, indicating that DNA synthesis and cell mass were not properly coupled. When the two strains were grown at 32°C, no significant difference in DNA-cell mass coupling could be detected (data not shown).

The growth rate of strain N1332 at the restrictive temperature was lower than that of strain N1331. Normally, when growth rate decreases, the DNA content per cell normally decreases also, but the DNA concentration (DNA per mass unit) will increase (6, 8). The average amount of DNA per cell for strain N1332 was higher and the DNA concentration was lower than for the wild type. Thus, in *recA* cells, DNA accumulation was perturbed in such a way that cells on the average contained more DNA than wild-type cells growing at the same rate. However, the *recA* cells were larger than *recA*$^+$ cells, resulting in DNA concentrations that were lower than in wild-type cells.

***recA*(Ts) strains at different temperatures.** To further characterize the possible role of RecA protein in DNA replication, strains N1331 and N1332 were grown and treated with RIF at different temperatures. The DNA histogram of N1332 after growth and RIF treatment at 32°C showed the same low frequency of cells with irregular numbers of chromosomes as the wild type (Table 4). Strain N1332 was then grown at 32°C and shifted to 42°C for 15 min to inactivate the RecA protein before RIF was added. The culture was split into two: one was kept at 42°C, and the other was shifted back to 32°C. After incubation with RIF at the two temperatures, the frequencies of cells with irregular numbers of chromosomes (Table 4) were 48% (42°C) and 24% (32°C). When strain N1331 was subjected to the same treatment, only minor changes in the expression of the asynchrony phenotype could be detected (Table 4). In addition, synchrony of initiation in constitutive *recA* mutants showed little temperature dependence (data not shown). An experiment in which N1332 cells were grown at the restrictive temperature for several generations before RIF addition and then incubated at 32 or 42°C gave similar results (data not shown), demonstrating that a 15-min incubation period at 42°C was sufficient to inactivate the RecA200 protein. Furthermore, it may be concluded that the RecA200 protein is to some extent renaturable. The fact that the asynchrony phenotype became less pronounced when the RecA200 protein was renatured after RIF addition shows that RecA exerts its effect on the chromosome configuration at a time point after the RIF-resistant stage of initiation.

DNA degradation. To assess the possible role of DNA degradation in determining the asynchrony phenotype, the maintenance of radioactively labeled DNA during runout of replication was measured. Only a moderate amount of degradation (about 15%) of the labeled DNA was detected after RIF treatment of the *recA* strains (Fig. 4). Most of the degradation occurred during the first 30 min of incubation, a

TABLE 3. Cellular DNA contents

Strain	Doubling time (min)	Avg DNA/cell[a]		Relative DNA concn[a] before RIF + FUZ	No. of chromosomes (% of cells)	
		Before RIF + FUZ	After RIF + FUZ		2, 4, or 8 chromosomes	3, 5, 6, or 7 chromosomes
JC2921	30	6.7	7.5	ND[b]	25	45
N1331	27	4.1	5.1	1.0	78	14
N1332	36	4.2	4.2	0.9	41	48

[a] Chromosome equivalents.
[b] ND, Not determined.

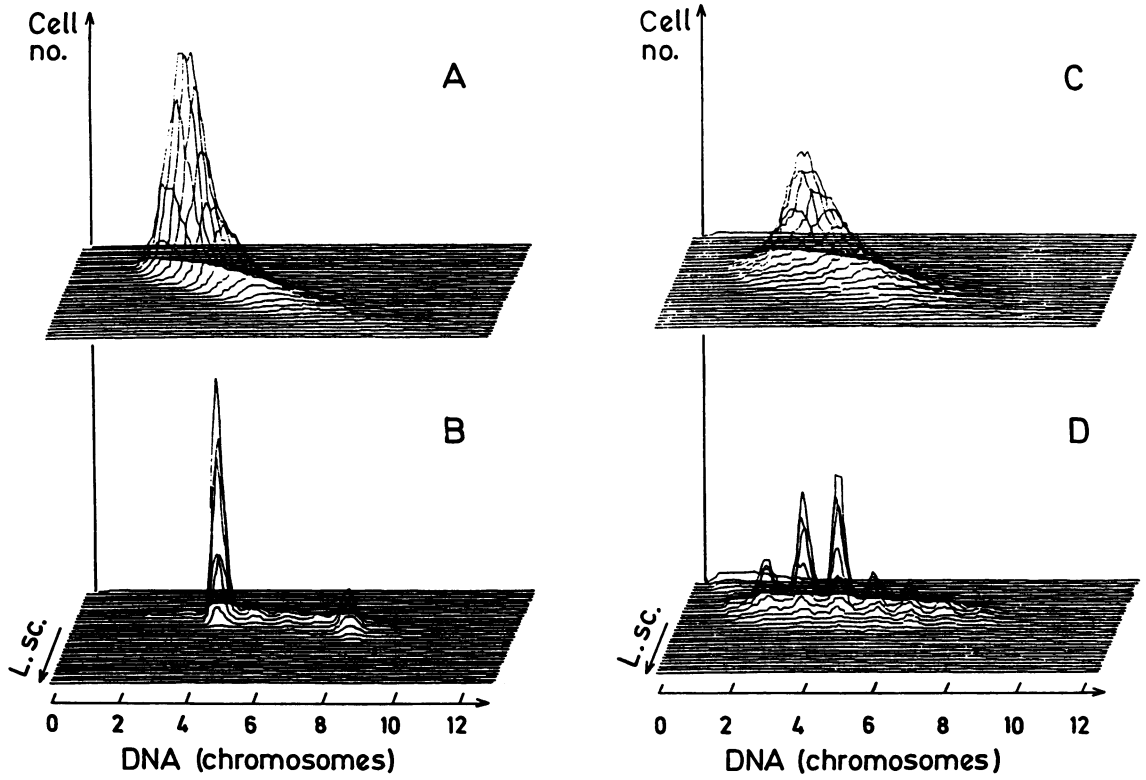

FIG. 3. DNA-light scatter (L.sc.) histograms of strains N1331 (A and B) and N1332 (C and D) growing exponentially at 42°C (A and C) and treated with RIF plus FUZ (B and D) at the same temperature.

time point at which these rapidly growing cells still contained multiforked chromosomal structures.

DISCUSSION

The present data show that recombination-deficient *recA* mutants do not initiate and/or complete DNA replication normally. After RIF treatment of such mutants, this property, which we have termed the asynchrony phenotype, can be visualized by DNA histograms showing major peaks at positions corresponding to three, five, six, and seven chromosomes. Since the vast majority of cells are found in peaks corresponding to integral numbers of chromosomes and since in no case have we seen evidence of peaks corresponding to half-chromosomes (1.5, 2.5, etc.), we conclude that the peaks represent cells with only whole, completed chromosomes. In the following we will consider three different models to explain the effect of the RecA protein on the asynchrony phenotype.

TABLE 4. Distribution of fully replicated chromosomes per cell after RIF treatment at different temperatures[a]

Growth conditions	Strain	No. of chromosomes (% of cells)							
		1	2	3	4	5	6	7	8
Growth at 32°C, RIF at 32°C	N1331	0	1	0	50	4	5	2	32
	N1332	0	0	0	36	2	4	3	40
Growth at 32°C, RIF at 42°C	N1331	0	2	1	65	5	6	2	15
	N1332	10	16	23	28	8	6	4	2
Growth at 32°C, 42°C for 15 min, RIF at 32°C	N1331	0	1	1	46	3	7	2	31
	N1332	0	2	3	38	5	9	7	24
Growth at 32°C, 42°C for 15 min, RIF at 42°C	N1331	0	1	0	38	3	4	2	43
	N1332	0	6	13	24	11	13	11	7

[a] Incubation in the presence of RIF was at the temperature indicated.

Degradation model. In this model, degradation of DNA is considered the only reason for the increased frequency of irregular numbers of chromosomes, meaning that individual chromosomes (fully replicated or not) are selectively and completely degraded while other chromosomes are left unaffected. From the data presented in Table 2, it is possible to estimate the amount of degradation necessary to create the observed effect. Assuming that all five-, six-, and seven-chromosome cells of strain JC2921 arose from cells that

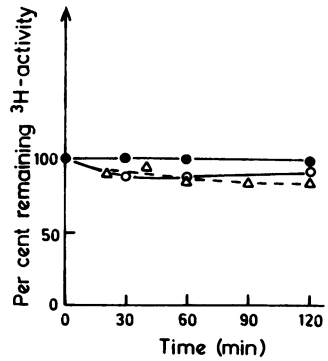

FIG. 4. Degradation of DNA labeled with [^{3}H]thymidine. Strains N1331 (●), N1332 (○), and JC2921 (△) were grown for two generations after labeling and then treated with RIF plus FUZ. The ordinate values were normalized to 100% at the time of drug addition for N1331 (11,900 cpm), N1332 (3,600 cpm), and JC2921 (2,270 cpm).

without degradation would have been eight-chromosome cells, about 17% of all chromosomes were lost. Since degradation will affect newly initiated as well as more extensively replicated chromosomes, the degradation necessary to account for the observed effect is considerably less and can be encompassed by the 15% degradation measured (Fig. 4). The same calculation may be performed for one-, two-, three-, and four-chromosome cells of strain N1332 (Table 2), giving about the same result. The magnitude of degradation measured is in reasonable agreement with the lack of increase in cellular DNA contents of strain N1332 during RIF runout compared with the 24% increase measured for strain N1331, both at 42°C.

During replication, both daughter chromosomes have one strand in common with the mother chromosome. It is difficult to envision how the daughter chromosomes can be totally degraded without affecting the contiguous mother chromosome. For this reason we do not favor the degradation model.

It may be assumed that degradation will increase if there are more free DNA ends inside the cells, for example, as a result of repair synthesis. To find out whether increased repair synthesis affected the asynchrony phenotype in *recA* cells, strain N1332 was grown at 42°C in the presence of 2-aminopurine (100 μg/ml). This is a base analog which is incorporated into DNA and shortly after replication is removed by mismatch repair (9). No difference could be found between histograms from *recA* cells grown in the presence and absence of 2-aminopurine (data not shown), suggesting that degradation is not responsible for the asynchrony phenotype. In an effort to decrease the extent of degradation, experiments were performed with *recA* mutants carrying additional mutations in *recB*, *recC*, or *recD* (and therefore lacking exonuclease V activity). Unfortunately, the double mutants were unable to carry out complete runout of replication, and no conclusions could be reached about their asynchrony phenotype.

Asynchrony model. One alternative explanation is that in *recA* strains, multiple initiations in each cell are distributed over the cell cycle so that the addition of RIF will catch a large fraction of the cells at a point when some but not all origins have been initiated. In contrast, the wild-type cell initiates all its origins within less than 10% of the cell cycle (24). We do not favor this model, because it has been shown that the variability in time between successive replications of a particular chromosome segment is about the same in wild-type and *recA* cells (12). It is not considered probable that the cell can lose coordination of initiations while maintaining control over the timing of initiations at each individual origin separately.

Abortive initiation model. The third model, which we favor, is that all initiations occur at the same time in the cell cycle but that a fraction of them are either left out or aborted during or shortly after initiation. This will result in cells containing three, five, six, or seven chromosomes in addition to cells with the normal four and eight chromosomes. Evidence for abortion of initiations or of the progress of replication forks close to *oriC* has been reported (1).

An estimate of the frequency of lost initiations may be given from the data presented in Table 2 as described above for the DNA degradation model. If five-, six-, and seven-chromosome cells are assumed to result from a loss of three, two, and one chromosome, respectively, the frequency will be about 17% of all initiations. Similar calculations for the two-, three-, and four-chromosome cells of strain N1332 (Table 4) give about the same result.

Two findings suggest that the RecA protein exerts its effect on initiation and replication over much of the cell cycle and not only during initiation. First, a large fraction of the N1332 cells contained irregular numbers of chromosomes after a 15-min incubation period at 42°C and subsequent RIF treatment. If initiations are properly coordinated, as they are in wild-type cells, less than 10% of the population will be initiating at any particular time at high growth rates (24) (Table 4). The fact that a considerably higher fraction of *recA*(Ts) cells had irregular numbers of chromosomes after a short incubation period at the nonpermissive temperature suggests that initiations may be lost for some time after the initiation event has occurred. Second, a reduction to 32°C after RIF addition reduced the fraction considerably, which means that a restoration of RecA activity after RIF addition also had an effect on cells that were not in the process of initiating. These findings are consistent with our model if it is postulated that in the absence of RecA protein, about 20% of the initiation complexes or newly formed replication forks are stalled or inactivated but can for some time be reactivated by the appearance of functional RecA protein.

The RecA protein has a role at the initiation stage of constitutive stable DNA replication (11), which is an alternative replication pathway occurring from origins other than *oriC* in the absence of RNase H (10, 31, 32). It is possible that the role of RecA protein in normal DNA replication is similar to that in constitutive stable replication, although in *oriC*-dependent initiation the RecA protein is not obligatory.

Conclusions. We have shown that in the absence of a functional RecA protein, the frequency of productive initiations is reduced about 20% compared with wild-type cells. Three models have been described to account for the observed data. In the preferred model, a defective RecA protein is proposed to stall the replication process during or shortly after initiation. However, we have no firm evidence to exclude a model involving specific and total degradation of newly made daughter chromosomes.

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