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Initiation of Chromosome Replication in *Escherichia coli* after Induction of *dnaA* Gene Expression from a *lac* Promoter

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***Escherichia coli* HB282 carries a *dnaA46*(Ts) allele on the chromosome, a wild-type *dnaA* allele under the control of the *lacUV5* promoter on the multicopy plasmid pBC32, and an overproducing *lac* repressor allele on an F′ factor. When the plasmid *dnaA* gene is repressed, the strain is thermosensitive. After a temporary deficiency in active *dnaA* protein at nonpermissive temperature, the addition of isopropyl-β-D-thiogalactopyranoside to the culture was found to produce a burst of initiations within 5 to 10 min at 30% of the origins in 90% of the cells. Initiations then continued at a rate slightly faster than the mass-doubling time such that after 2 h the origin-to-mass ratio of the control culture was restored.**

The *dnaA* protein in *Escherichia coli* is required during an early step in the initiation of chromosome replication (1, 5, 14) and has been implicated in the control of the timing of replication (7–9, 12). We previously reported the construction of a plasmid which carries a wild-type *dnaA* allele under the control of the *lac* promoter (4). In a host with a thermosensitive *dnaA* allele on the chromosome, induction of synthesis of active *dnaA* protein from the plasmid at the nonpermissive temperature was found to produce an immediate increase in the rate of DNA accumulation (4). In a *dnaA*$^+$ strain, however, induction of additional *dnaA* protein synthesis from the plasmid did not stimulate DNA synthesis. This confirmed previous suggestions that *dnaA* protein might not normally be a limiting factor for chromosome replication (6, 10, 11). Those experiments were done with a *recA* strain which rendered the DNA unstable during inhibition of DNA synthesis and permitted only qualitative measurements of DNA accumulation, not of initiation (4). Here we used a *recA*$^+$ strain to examine the kinetics of the *lac*-induced replication after initiation had been halted for some time at the nonpermissive temperature. Would the induced initiations occur in a single step at all origins? Could there be multiple initiations per origin (more than one initiation per origin per mass-doubling time), or would initiations occur only gradually?

The expression of *dnaA* genes from the plasmid was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to cultures kept for periods of 10 to 35 min at 42°C. Samples of cultures were incubated for 60 min in the presence of rifampin before acid precipitation and DNA assay. Rifampin prevents further initiations without affecting the completion of ongoing rounds of replication (3, 8, 14), such that the final amount of DNA accumulated becomes a measure for initiation and for the number of replication origins present at the sampling time. Under the conditions used, rifampin given at 30°C caused DNA accumulation to cease within 60 min (1.52-fold increase); the DNA plateau remained constant during further incubation. The plateau was slightly lower than that produced by a shift to 42°C (1.58-fold increase), suggesting that rifampin stops initiation somewhat faster than does a shift to the nonpermissive temperature. The initial rate of initiation reached maximum levels at 1 mM IPTG, although culture and colony growth at 42°C were normal at 20-fold-lower IPTG concentrations (not shown).

When *dnaA* expression from the plasmid was induced after initiation had been halted for some time at the nonpermissive temperature, a small burst of nearly synchronous initiations occurred within the first 5 min after IPTG addition. This was followed by a roughly exponential increase in the rate of initiation with a doubling time similar to that of the culture growth. Initiation was, however, slightly faster than mass accumulation; after 2 h, the control culture, which had received IPTG at zero time, and the portion which had received IPTG 25 min later, had essentially the same number of origins per unit of cell mass (Fig. 1). In this experiment, the cultures had been periodically diluted to keep growth exponential. From several repeats of such experiments (using shorter sampling intervals for a shorter total time), it appeared that the approach of the origin kinetics of the culture with the temporary initiation stop to the control curve occurred in small steps about one mass-doubling time apart, rather than gradually. The curve in Fig. 1 has been drawn through the measuring points accordingly, although these steps are too small to be demonstrated clearly by the method used. The initial burst of initiations resulted in about a 30% increase in the number of origins, which was found to be independent of the duration (15 to 35 min) of the period without initiations. The induction never reached the control curve in one step, even when IPTG was given after 10 min.

A conceivable interpretation of this result would be that *dnaA* protein activity has to be present during a certain period of the cell cycle, corresponding to a 30% "window," to be effective. In that case, initiations from the initial burst should occur at all origins in 30% of the cells. To see whether the observed burst of initiations occurred at 100% of the origins in 30% of the cells or at 30% of the origins in 100% of the cells, the experiment was repeated (IPTG after 35 min), and the DNA was radioactively pulse-labeled 5 min after the induction by IPTG. The presence or absence of DNA synthesis in the cells was then visualized by autoradiogra-

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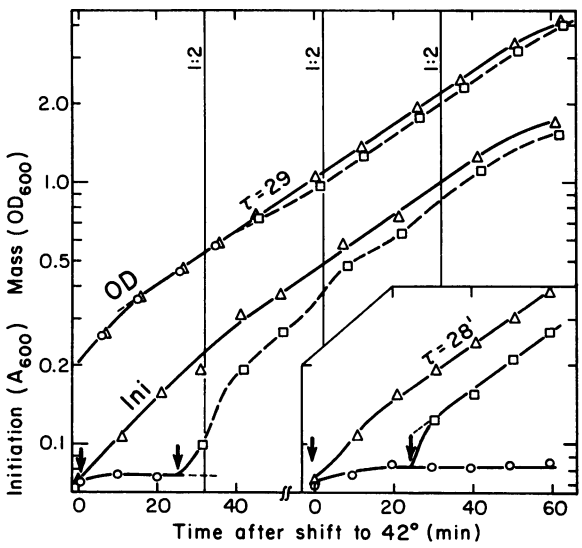

FIG. 1. Initiation of replication by induction of *dnaA* expression from the *lac* promoter. Strain HB282 (4) was grown at 30°C in LB medium (13) containing 2 mM $MgSO_4$ to reduce a tendency to lyse in the presence of rifampin. At an optical density at 600 nm (OD_{600}) of 0.2, the culture was shifted to 42°C and divided into three portions; the first one received 1 mM IPTG immediately (Ini) (△), the second portion received 1 mM IPTG 25 min later (□), the third portion received no IPTG (○). To keep the growth exponential, the culture was periodically diluted twofold as indicated. Samples of 5 ml each were added to rifampin (final concentration, 0.15 mg/ml) and further incubated for 60 min with aeration at 42°C. The DNA in these samples was assayed colorimetrically (2) as a measure for initiation. The inset is of a similar experiment showing more clearly the initial step, corresponding to a 30% increase in the number of origins.

phy. Counts of several microscope fields showed that 90% of the cells were labeled (not shown); i.e., the second alternative was correct. Thus, there is no reason to assume a cell cycle window for *dnaA* protein activity.

Why is it that in those cells in which active *dnaA* protein was lacking at the time initiation should have occurred, the belated supply of *dnaA* protein allowed only a fraction of the origins in every cell to "fire"? One possible explanation would be that the induced synthesis of *dnaA* protein from the plasmid is insufficient to keep up with the increased demand when all chromosomal *dnaA* protein is inactivated. We believe that this is unlikely, because even a 20-fold-lower inducer concentration (0.05 mM) was sufficient to produce normal initiation and colony growth at 42°C. Only the initial burst of initiations was delayed for a few minutes at this lower concentration after a prolonged halt in initiations.

As an alternative explanation, we suggest that some of the origins in the preinitiation stage cannot be immediately activated by active *dnaA* protein synthesized de novo from the plasmid because inactive *dnaA* protein of chromosomal origin is present in the initiation complex, which blocks the access of the active protein. This inactive *dnaA* protein is later replaced by active protein, thus explaining the slow approach to the control curve. This interpretation is consistent with results of experiments in which the temperature was shifted down again to 30°C at the same time that IPTG was given. In this case, the initial burst was found to be about twice as high as the one without the temperature downshift, corresponding to 50 to 60%, although never to 100%, of the origins (not shown). In the case of a temperature downshift, IPTG could be omitted with essentially the same result (only slightly lower increase). Thus, under our conditions, the heat inactivation of *dnaA* protein was not completely reversible. This interpretation can be tested in the future by constructing a strain in which the chromosomal *dnaA* gene is deleted, such that replication depends entirely on the synthesis of plasmid-derived *dnaA* protein, whose synthesis can be turned on and off without possible interference from inactive protein or from temperature shift effects.

The fact that the number of origins increased faster than cell mass means that some origins replicated twice during one mass-doubling time. Are these extra initiations after induction by IPTG controlled by a "pacemaker" (6) which does not change because of a temporary *dnaA* protein deficiency, or do these initiations reflect a random approach to the control origin-to-mass ratio? The doubling time of the origin curves, obtained when IPTG was added a short time after a shift to 42°C, was essentially the same as that of the control (Fig. 1, inset). This leads us to favor the first alternative and suggests that, after a temporary deficiency in active *dnaA* protein, initiations can occur at the correct time despite a reduced origin-to-mass ratio.

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