JOURNAL OF BACTERIOLOGY, July 1989, p. 3591–3596
0021-9193/89/073591-06$02.00/0
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Vol. 171, No. 7

Inhibition of Protein Synthesis Transiently Stimulates Initiation of Minichromosome Replication in *Escherichia coli*

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Received 7 November 1988/Accepted 28 March 1989

Replication of *oriC*-dependent minichromosomes was found to be transiently stimulated when protein synthesis was inhibited by the addition of chloramphenicol. Initiation of replication was also induced by amino acid starvation of *relA* mutant strains and a nutritional upshift. The results are explained on the basis that these treatments rendered RNA polymerase more available for participation in the initiation process. As a consequence, the *oriC* duplex may be transcriptionally activated to an open form, a necessary prerequisite for DNA polymerization.

Initiation of chromosome replication at *oriC*, the unique origin of replication of the *Escherichia coli* chromosome, is the limiting step which governs the overall rate of both DNA replication and cell proliferation (12, 44). Participating early in the initiation process at *oriC* are the products of the *dnaA*, *dnaB*, and *dnaC* genes, HU protein, and RNA polymerase. By use of an in vitro replication system, the functions of these proteins in the biochemistry of initiation have been defined, but their roles, if any, in the timing of the initiation reaction have yet to be determined (16, 26). The DnaA protein is a candidate for a timing element because it is absolutely required for initiation at *oriC* and acts early in the reaction to open the *oriC* duplex prior to the start of DNA polymerization (4). Furthermore, overexpression of the *dnaA* gene product leads to induction of initiation from *oriC* on both chromosomes and minichromosomes (1, 31; O. Pierucci, personal communication). This finding is consistent with the DnaA protein's being limiting for initiation, but it also raises a question: Can an excess of any of the other participants in the earliest stages of the reaction also stimulate initiation? We chose to examine RNA polymerase because it may act to transcriptionally activate the origin, functioning as the DnaA protein, to open the *oriC* region.

Addition of the protein synthesis inhibitor chloramphenicol to a culture is a convenient means to alter the pattern of transcription by RNA polymerase in *E. coli*. Almost immediately after chloramphenicol addition, functioning enzyme is preferentially redistributed to stable RNA promoters (37). In addition, the rate of transcription is increased in a growth rate-dependent fashion, as a result of an increase in the fraction of RNA polymerase molecules involved in transcription. Therefore, although the addition of chloramphenicol ultimately leads to an inhibition of initiation events at *oriC*, the availability of RNA polymerase to function in the initiation reaction would be increased by this method. Similarly, amino acid starvation of *relA* mutant cells and a nutritional upshift duplicate the chloramphenicol-induced redistribution of RNA polymerase molecules and the ensuing changes in transcription due to altered activity or availability of RNA polymerase (5, 8, 18, 27, 38). All three procedures have been shown to induce initiation at *oriC* in temperature-sensitive *dnaA* mutants growing at temperatures above the permissive temperature (10, 17, 25, 30, 40).

The role of elevated RNA polymerase activity in initiation of minichromosome replication in $dnaA^+$ cells was assessed by chloramphenicol addition, amino acid starvation of *relA* mutant cells, and a nutritional upshift. This sensitive replication assay was used to monitor the effects of the treatments and facilitated detection of small changes in the rate of *oriC*-dependent initiation of DNA replication. An abrupt, transient stimulation of initiation from extrachromosomal copies of *oriC* was detected immediately after each of the treatments.

MATERIALS AND METHODS

Bacteria, plasmids, and growth conditions. Most experiments were performed with a *recA* derivative of *E. coli* K-12, MM294 (*endA thi hsdR17 supE44*). This strain was obtained from the Coli Genetic Stock Center. The *recA1* allele was introduced into MM294 by mating with KL16-99. On some occasions *E. coli* B/r F (*thyA his*) was used in place of MM294. CP78 (*thr-1 leuB6 his-65 argH46 thi-1 ara-13 gal-3 malA1 xyl-7 mtl-2 tonA2 supE44* $relA^+$) and CP79 (*thr-1 leuB6 his-65 argH46 thi-1 ara-13 gal-3 malA1 xyl-7 mtl-2 tonA2 supE44 relA*) were obtained from E. Morgan. Minichromosomes employed were pAL49 (7.6 kilobases [kb], $mioCp^+$) (20) and pAL4 (7.0 kb, $mioCp^-$) (21). Other plasmids used were pSC101 (7), pBR322 (3), pACYC184 (6), and pML31 (24). Cultures were grown at 37°C in minimal salts medium (20) supplemented with glycerol (0.1%), glucose (0.1%), or glucose (0.1%) plus casamino acids (0.2%). Required amino acids were added at 50 μg/ml. Thiamine and thymine were added at 10 μg/ml. Final concentrations of 15 μg of tetracycline per ml and 25 to 100 μg of kanamycin per ml were present in cultures containing plasmids specifying resistance to these drugs. Chloramphenicol was added by transferring a portion of a culture to a prewarmed flask containing the drug. Chloramphenicol had been added to the flask as an ethanol solution, and the solvent was evaporated under vacuum prior to the addition of cells. Amino acid starvation (5) and nutritional shifts (17) were effected by adding a portion of a culture to appropriate amounts of prewarmed valine (5 mg/ml) and 20% casamino acids to produce final concentrations of 0.5 mg/ml and 3.3%, respectively.

Measurement of plasmid replication. A whole-cell lysis protocol was used to measure plasmid replication (20).

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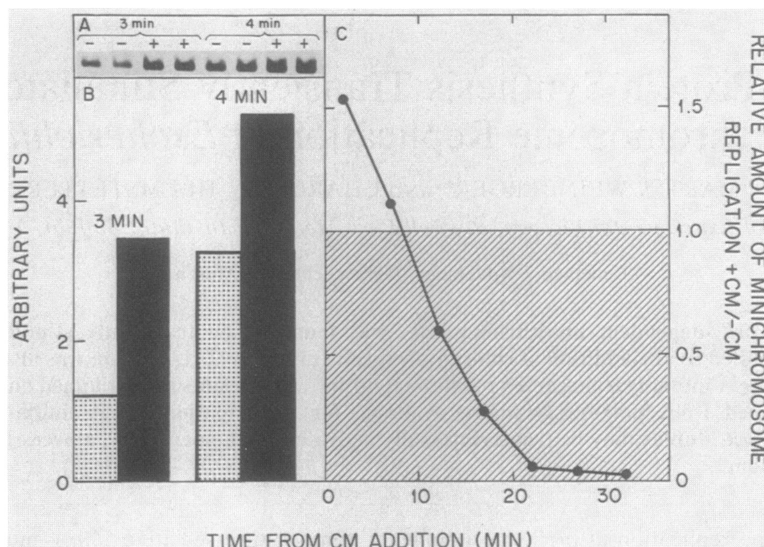

FIG. 1. Effect of chloramphenicol (CM) addition to *E. coli* MM294 *recA*(pAL49). (A) Duplicate 0.5-ml portions of MM294 *recA*(pAL49) growing in glucose minimal medium were pulse-labeled with 30 μCi of [^{3}H]thymidine per ml for 3 and 4 min in the presence (+) and absence (−) of 200 μg of chloramphenicol per ml. At the end of the labeling period, 50 μl of a 1-mg/ml thymidine solution was added, and the samples were incubated for an additional 2 and 1 min, respectively, so that the period of exposure to label was 5 min in all cases. Then, 0.1 volume of ice-cold 1 M NaN_3 was added, and extracts were prepared. A total of 200 kcpm from each extract was electrophoresed and fluorographed as described in the text. Shown are the minichromosome bands present on the fluorogram. (B) Densitometry was used to measure the intensity of minichromosome bands in panel A. Presented are the average values of the duplicate samples in the presence of chloramphenicol (■) and in the absence of chloramphenicol (□). (C) A culture of MM294 *recA*(pAL49) growing in glucose minimal medium was divided in two at time zero, and one portion was brought to 200 μg of chloramphenicol per ml. Every 5 min, 10-ml samples from both chloramphenicol-treated and drug-free control cultures were pulse-labeled with 10 μCi of [^{3}H]thymidine per ml for 4 min. Incorporation was terminated, samples were held at 0°C, and extracts were prepared, electrophoresed, and fluorographed as described in the text; 500 kcpm of each extract was examined. The ratio of the relative amounts of minichromosome replication in the presence and absence of the drug is plotted with respect to the time of chloramphenicol addition. Each data point represents the midpoint of the period of labeling. The portion of the curve above the shaded area represents stimulated minichromosome replication (ratio with and without chloramphenicol [+CM/−CM] > 1), and the portion in the shaded area represents inhibition of minichromosome replication (+CM/−CM < 1).

Samples (0.5 to 10 ml) were pulse-labeled with 4 to 30 μCi of [*methyl*-^{3}H]thymidine (70 to 80 Ci/mmol; New England Nuclear Corp.) per ml. Incorporation was terminated by addition of 100 μg of unlabeled thymidine per ml and 0.1 volume of 1 M NaN_3. Cells were held at 0°C and then lysed, and the amount of radioactivity incorporated in each extract was determined. In all experiments, a constant amount of radioactivity from each sample was applied to an agarose gel. Electrophoresis, fluorography, and quantitation of plasmid DNA by densitometry (Bio-Rad model 620 video densitometer) were performed as described before (20).

RESULTS

Effect of chloramphenicol on minichromosome replication. When chloramphenicol was added to a minichromosome-containing culture, minichromosome replication was stimulated. Samples from a glucose-grown culture of MM294 *recA*(pAL49) were brought to 200 μg of chloramphenicol per ml and pulse-labeled with [^{3}H]thymidine. As shown in Fig. 1A, the drug stimulated minichromosome replication. The extent of minichromosome replication seen in Fig. 1A was quantitated by densitometry (Fig. 1B). Addition of the drug increased minichromosome replication 2.88- and 1.62-fold at 3 and 4 min, respectively. The effects of continued exposure to chloramphenicol on minichromosome replication are shown in Fig. 1C. Drug was added at time zero, and sequential samples were removed and pulse-labeled with [^{3}H]thymidine. Relative to the drug-free control, drug treatment stimulated replication of minichromosome DNA for 10 min. At later times, incorporation of label into minichromosomes was inhibited, reaching a minimum of 2% of that into the untreated control at 30 min.

Stimulation of minichromosome replication in the absence of protein synthesis was investigated further and revealed the following. (i) Drug-induced replicative stimulation of minichromosomes in *E. coli* B/r strains occurred at lower concentrations of chloramphenicol than in K-12 strains. Maximum stimulation in B/r strains was achieved with the addition of 50 μg of chloramphenicol per ml, and enhanced minichromosome replication was not detected with addition of more than 100 μg of chloramphenicol per ml. (ii) Detection of enhanced minichromosome replication required a minimum of 3 min of exposure to the drug. (iii) Identical replicative responses to chloramphenicol addition were observed in strains containing pAL49 (*mioCp*$^+$) and pAL4 (*mioCp*$^-$). (iv) Chloramphenicol-stimulated minichromosome replication was seen in glucose and glycerol minimal media. Addition of the drug did not stimulate minichromosome replication in cells grown in a richer (glucose-casamino acids) medium. (v) Chloramphenicol-enhanced minichromosome replication was *recA* independent. Addition of drug to *recA* and *rec*$^+$ strains resulted in equivalent replicative stimulation.

Effect of amino acid starvation on minichromosome replication. Amino acid starvation of a *relA* strain and chloramphenicol addition to a *rel*$^+$ strain produce similar changes in transcription patterns (5, 18, 27). Therefore, it was of interest to see whether amino acid starvation of a *relA* strain

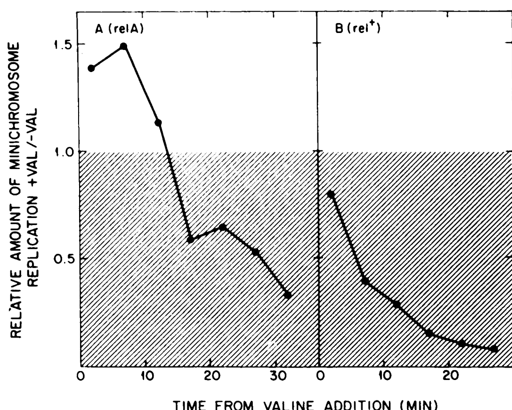

FIG. 2. Effect of valine (VAL) addition to *E. coli* CP78(pAL49) and CP79(pAL49). Cultures of (A) CP79 *relA*(pAL49) and (B) CP78 *rel*$^+$(pAL49) growing in glucose minimal medium were divided in two at time zero, and one portion of each culture was brought to 0.5 mg of valine per ml. Every 5 min, 2.5-ml samples from valine-supplemented and untreated cultures were pulse-labeled with 20 μCi of [^{3}H]thymidine per ml for 4 min. Extracts were prepared, and 125 kcpm of each extract was electrophoresed and fluorographed as described in the text. The relative amounts of minichromosome replication in the presence and absence of valine are plotted with respect to the time of valine addition. Data are presented as in Fig. 1C.

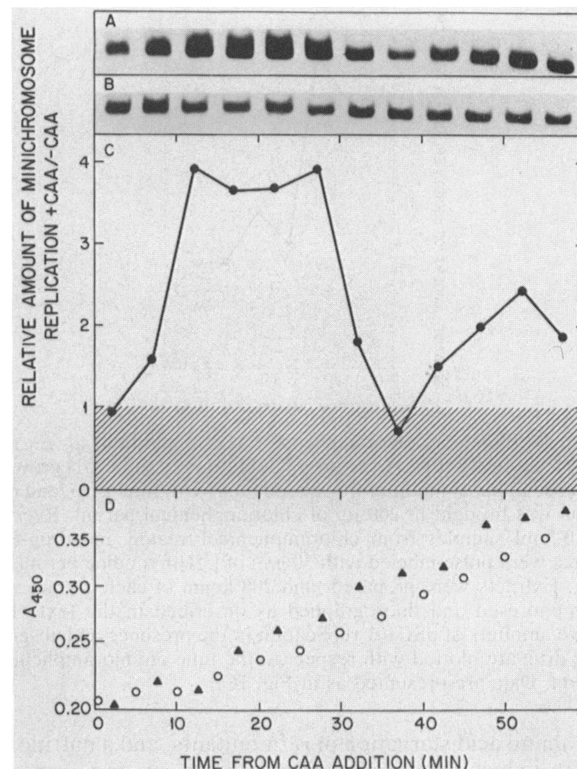

FIG. 3. Effect of a nutritional shift on *E. coli* MM294 *recA*(pAL49). A culture of MM294 *recA*(pAL49) growing in glucose minimal medium was divided in two at time zero. One portion of the culture was brought to 3.3% casamino acids (CAA). Every 5 min, 1.0-ml samples from shifted and unsupplemented cultures were pulse-labeled with 20 μCi of [^{3}H]thymidine per ml for 4 min. Extracts were prepared, and 125 kcpm of each extract was electrophoresed and fluorographed as described in the text. Shown are pAL49 bands present on the fluorogram in the presence (A) and absence (B) of casamino acids. (C) Relative amounts of minichromosome replication in the presence and absence of casamino acids, plotted with respect to the time of the nutritional shift. Data are presented as in Fig. 1C. (D) A_{450} of shifted (▲) and unshifted (○) cultures as a function of time after the addition of casamino acids.

would also stimulate minichromosome replication. The minichromosome pAL49 was introduced into isogenic *relA* (CP79) and *rel*$^+$ (CP78) strains. Amino acid starvation, induced by the addition of valine (final concentration, 0.5 mg/ml), stimulated pAL49 replication only in the *relA* strain (Fig. 2). The kinetics of minichromosome replication in valine-supplemented *relA* cells were somewhat different from those seen after the addition of chloramphenicol. At 5 min after the addition of valine, minichromosome replication was stimulated 1.5-fold relative to the untreated sample. The subsequent inhibitory effect of valine addition on minichromosome replication was less pronounced than seen with chloramphenicol addition.

Effect of nutritional shift on minichromosome replication. RNA polymerase availability is also altered by a nutritional shift (8, 38). The effect of casamino acid supplementation (final concentration, 3.3% [18]) on minichromosome replication in a glucose-grown culture of MM294 *recA*(pAL49) is shown in Fig. 3A and B. In Fig. 3C, the relative amount of minichromosome replication in the presence and absence of casamino acids is plotted as a function of time after the nutritional shift. pAL49 replication was slightly elevated at 5 min, but maximum stimulation was not achieved until 10 min after the shift. Between 10 and 25 min postshift, minichromosome replication was approximately fourfold greater in casamino acid-treated cells than in the unshifted control. Minichromosome replication then decreased rapidly to less than the control value at 35 min. This decrease was followed by a second interval of elevated pAL49 replication.

Following casamino acid addition, the rates of mass increase of shifted and control cultures were monitored by measuring the absorbances of the cultures (Fig. 3D). The nutritional shift did not change the rate of increase in absorbance for the first 60 min, presumably due to the relatively high concentration of casamino acids used (final concentration, 3.3%). The stimulation of minichromosome replication was therefore not attributable to a nutritional acceleration of the rate of total cellular mass synthesis. Supplementation of a glucose-grown culture with 0.2% (final concentration) casamino acids resulted in the anticipated (1.5-fold) change in the rate of mass accumulation and an abrupt 2-fold increase in minichromosome replication. At 25 min postshift, the rate of minichromosome replication declined to 1.5-fold over the preshift rate, where it remained (data not shown).

Effect of chloramphenicol on other plasmids. Chloramphenicol did not induce an early replicative stimulation of the high-copy-number plasmids pBR322 and pACYC184. Enhanced replication of plasmids with lower copy numbers (e.g., pSC101 and pML31 [mini-F]) was induced by chloramphenicol addition. As shown in Fig. 4, addition of chloramphenicol increased the rate of pSC101 replication over threefold for 15 min. The extent of replicative stimulation subsequently decreased but remained in excess of the control value at 30 min.

DISCUSSION

RNA polymerase availability. Initiation of minichromosome replication was stimulated by treatments that alter the intracellular pattern of transcription. Chloramphenicol addi-

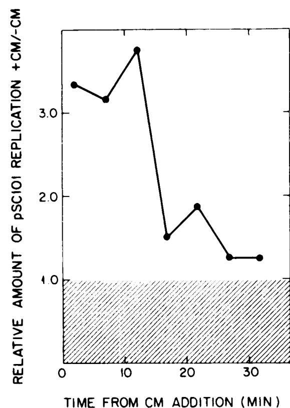

FIG. 4. Effect of chloramphenicol (CM) addition to *E. coli* MM294 *recA*(pSC101). A culture of MM294 *recA*(pSC101) growing in glucose minimal medium was divided in two at time zero, and one portion was brought to 200 μg of chloramphenicol per ml. Every 5 min, 0.5-ml samples from chloramphenicol-treated and drug-free cultures were pulse-labeled with 30 μCi of [^{3}H]thymidine per ml for 4 min. Extracts were prepared, and 200 kcpm of each extract was electrophoresed and fluorographed as described in the text. The relative amounts of pSC101 replication in the presence and absence of the drug are plotted with respect to the time of chloramphenicol addition. Data are presented as in Fig. 1C.

tion, amino acid starvation of *relA* mutants, and a nutritional upshift induce pleotropic effects but have one common feature: an immediate redistribution of preexisting RNA polymerase activity (8, 18, 27, 37, 38). The redistribution is a result of the combined effects of decreased frequencies of initiation of transcription at some promoters and recruitment of previously sequestered (nonsynthetic) molecules (19). The redistribution increases the availability of the enzyme to function at available promoters. One consistent and clearly evident manifestation of this redistribution is the rapid (<1 min) two- to threefold stimulation in the rate of initiation of stable RNA transcripts (37). Although the enhanced replication from *oriC* induced by the three treatments may not directly parallel changes in transcription, we suggest that the observed changes in replication are also consequences of increased RNA polymerase availability.

A precedent for a change in the availability of RNA polymerase leading to initiations from *oriC* is the behavior of conditional initiation-defective *dnaA* mutant strains, in which *dnaA* activity limits the rate of initiation at *oriC*. Bursts of DNA synthesis corresponding to initiation of chromosome and minichromosome replication were induced under conditions that would be expected to increase the availability of RNA polymerase (10, 17, 25, 30, 40). In the present study, analogous RNA polymerase-mediated effects were seen in *dnaA*$^+$ strains.

Chloramphenicol-induced replicative stimulation. Earlier work indicated that inhibition of protein synthesis blocked initiation of new rounds of chromosome replication from *oriC* but that ongoing rounds continued to completion (12, 44). Using the much more sensitive minichromosome system, which is capable of detecting small changes in initiation frequency, we found that chloramphenicol addition results in transient stimulation of initiation at *oriC*, prior to the well-established inhibitory effects of the drug. It has previously been reported that chloramphenicol inhibited minichromosome replication in a glucose-casamino acid–grown culture only after a delay of 5 to 7 min (45). This effect probably resulted from the summation of an immediate, brief stimulation of replication and a subsequent inhibitory effect. In richer media, drug-induced alterations in transcription are reduced relative to that in poorer media (27, 37) and may account for the lack of a demonstrable replicative stimulation in glucose-casamino acids medium.

Leftward transcription into *oriC* initiated at *mioCp* is under stringent control in vivo and in vitro and negatively regulated by the *dnaA* gene product (14, 23, 34; reviewed by A. C. Leonard and C. E. Helmstetter [*in* K. W. Adolph, ed., *Chromosomes: Eukaryotic, Prokaryotic and Viral*, in press]). Because chloramphenicol addition stimulates transcription initiating at *mioCp* and *oriC* function is inhibited by transcription into the origin from a *lacp* inserted adjacent to *oriC*, it was proposed that chloramphenicol-mediated inhibition of *oriC* function could be related, in part, to increased transcription into the origin (32, 39). This interpretation is at variance with the drug-induced replicative stimulation we have observed. The fact that *mioCp* is dispensable for the replication of chromosomes (43) and minichromosomes (11, 20) precludes assigning a definitive role in the initiation process to transcripts entering *oriC* from this promoter.

Drug-induced stimulation of replication associated with an altered availability of RNA polymerase was not limited to minichromosomes. Addition of chloramphenicol to pSC101-containing cultures enhanced replication of this *dnaA*-dependent replicon in a fashion analogous to minichromosomes. The lack of chloramphenicol-mediated replicative stimulation of the high-copy-number plasmids pBR322 and pACYC184 can be explained by the observation that increased availability of RNA polymerase does not alter the rates of synthesis of the transcripts (RNA I and RNA II) which regulate the replication of these replicons (22).

Replicative stimulation in a relaxed strain. Amino acid starvation of a *relA* strain also stimulated minichromosome replication in the absence of protein synthesis. Amino acid starvation of *relA* cells is analogous to chloramphenicol treatment of *rel*$^+$ cells. In both cases, ppGpp, the effector of the stringent response, does not accumulate, transcription of primarily stable transcripts is enhanced (5, 18, 27, 37), and *oriC* function, as measured by minichromosome replication, is stimulated. We suggest that the stringent response was involved in replicative stimulation only to the extent that amino acid starvation of *relA* cells altered the availability of RNA polymerase. In *dnaA* mutants (30) and in *oriC*-independent stable DNA replication (42), alterations in the activity of RNA polymerase induced by amino acid starvation of *relA* mutant cells also lead to enhanced replication. Our findings differ markedly from those in an earlier report, that initiation of chromosome replication in *E. coli* continued for a period of hours during amino acid starvation of a *relA* strain (A. Jimenez-Sanchez, E. C. Guzman, and F. J. Carrillo, Abstr. EMBO Workshop, Molecular Basis of Bacterial Growth and Division, 1987, p. 181). This extended *rel*-dependent replication clearly differs from the transient stimulation we have described.

In *Bacillus subtilis*, it is possible to remove amino acids from a *relA* strain without causing the alteration in the pattern of transcription usually associated with amino acid starvation. When this was done, initiation of chromosome replication was seen to be under stringent control (35). There are fundamental differences between the origin regions of *E. coli* and *B. subtilis*, i.e., the presence of an rRNA operon adjacent to the *B. subtilis* origin of replication and the existence of an RNA-DNA copolymer complementary to this rRNA (13, 36). The stringent control of initiation of chromosome replication in *B. subtilis* is therefore seen as

distinct from the stringency-related replicative stimulation observed in *E. coli*.

Effect of nutritional shift. In addition to redistributing RNA polymerase activity, addition of chloramphenicol to *rel*$^+$ cells and amino acid starvation of *relA* cells block protein synthesis and are, as a consequence, ultimately inhibitory to cell growth. Due to this interruption of protein synthesis, these treatments produce only a short-term enhancement of replication from *oriC*. A nutritional upshift increases the availability of RNA polymerase in the absence of such inhibitory effects, maximizing the replicative stimulation attributable to increased RNA polymerase availability. It is important to note that the abrupt increase in minichromosome replication caused by the nutritional upshift could not have been due to a change in the rate of cell mass synthesis, because this rate was unchanged for the first 60 min after the shift. Even if it had been due to an increase in the rate of mass synthesis (12, 44), the rate of minichromosome replication would be expected to increase twofold, at most, under these conditions (44), rather than fourfold, as reported here.

Role of RNA polymerase in initiation. There are three possible roles for RNA polymerase in the process of initiation of DNA synthesis. The first is production of a transcript which functions as a primer for replication of the leading strand. In *B. subtilis*, RNA polymerase appears to synthesize such a transcript (36), but it is not clear whether RNA polymerase or primase (*dnaG* gene product) is responsible for in vivo primer synthesis in *E. coli*. In a reconstituted in vitro system, primer for leading-strand synthesis can be provided by RNA polymerase alone (15), by a combination of RNA polymerase and primase (29), or by primase alone (41). The greater specificity of *dnaG*-mediated priming suggests that the role of RNA polymerase is not related to priming. Recently, in vitro studies have indicated that the role of RNA polymerase in initiation of replication involves opening of the template molecule (4).

The second possible role for RNA polymerase is synthesis of (nonpriming) transcripts which could be involved directly or after translation into protein in the process of initiation. Examples of the former would include regulatory RNA molecules involved in replication of some plasmids (28), but there is no experimental evidence for the direct involvement of RNA molecules in initiation of DNA synthesis at *oriC*. There are numerous examples of the latter, translation-dependent alternative, but it can be eliminated as an explanation for the effects reported here because chloramphenicol addition would block synthesis of such positive-acting proteins.

The third possible role for RNA polymerase is transcriptional activation of the origin, in concert with other components of the initiation complex (16, 26, 29, 45). In other replicons, transcriptional activation is a key step in the process of initiation of replication (9). Evidence has recently been obtained that the role of RNA polymerase in initiation of replication at *oriC* involves a transcriptional activation step (2). The replicative stimulation associated with altered availability of RNA polymerase can be best explained on the basis of transcriptional activation of *oriC* by an RNA polymerase-containing initiation complex, based on the following reasoning. Increased availability of RNA polymerase is not unique in producing elevated levels of *oriC*-dependent initiation events. Overexpression of a defective *dnaA* gene product can result in suppression of its thermosensitive phenotype (32), and overexpression of wild-type *dnaA* gene product yields increased initiations at *oriC* (1, 31; O. Pierucci, personal communication). Thus, increased levels of both the *dnaA* gene product and RNA polymerase can stimulate initiation of DNA replication from *oriC*, but both components are not likely to be simultaneously limiting for initiation of replication at *oriC* during steady-state growth. It has been suggested that an excess of one initiation factor may compensate for the deficiency of another (D. W. Smith and J. W. Zyskind, *in* K. W. Adolph, ed., *Chromosomes: Eukaryotic, Prokaryotic and Viral*, in press). This may be especially true if both factors function very early in the process and can participate in formation of the same or similar end products. In this instance, the end product appears to be formation of an open complex at *oriC* prior to the start of DNA polymerization (2, 4). If they acted synergistically, increasing the amounts of any of the key early components might drive the assembly and/or activation of the replisome complex. If formation of the open complex normally depended on the binding of a fixed amount of DnaA to *oriC*, then it seems likely that the synergism between RNA polymerase and DnaA protein in function at *oriC* could allow increased amounts of RNA polymerase to cause initiation to take place before the requisite amount of DnaA protein is bound.

ACKNOWLEDGMENTS

We thank Olga Pierucci and Alan Leonard for helpful discussions.
This investigation was supported by Public Health Service grant GM26429 from the National Institute of General Medical Sciences.

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