Differential inhibition of the initiation of DNA replication in stringent and relaxed strains of *Escherichia coli*

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Summary

Starvation for isoleucine inhibits chromosome, minichromosome and pBR322 DNA replication in a stringent strain of *E. coli*, but does not do so in a relaxed mutant. Starvation for other amino acids inhibits either chromosome and minichromosome replication in both strains. From these results we conclude that *oriC* and pBR322 replication are stringently regulated and that isoleucine seems not to be essential for the protein synthesis required at the initiation of *oriC* replication. Deprivation of isoleucine in a Rel⁻ strain gives rise to amplification of minichromosome and pBR322 with a better yield of the latter plasmid than that following treatment with chloramphenicol.

1. Introduction

Initiation of chromosome replication requires RNA and protein synthesis (Maaloe & Hanawalt, 1961; Messer, 1972) and inhibition of the synthesis of either of these macromolecules has long been used to inhibit the initiation step without affecting elongation (Bremer & Churchward, 1977, Maaloe & Hanawalt, 1961). RNA synthesis can be inhibited specifically by the use of one of several drugs which inhibit RNA polymerase activity, but the inhibition of protein synthesis by amino-acid deprivation also affects RNA synthesis through ppGpp accumulation, and induction of the stringent response (Cashel, 1975; Gallant, 1979). Thus inhibition of the initiation of chromosome replication by amino acid starvation could be a result of RNA synthesis inhibition via the stringent response if the synthesis of an RNA required for initiation were stringently regulated.

In this work we asked if the synthesis of an RNA required for the initiation of *E. coli* chromosome replication is inhibited by the stringent response. For this purpose we compared the replication of the minichromosome pSY317 the plasmid pBR322 and the bacterial chromosome. We observed that starvation for isoleucine, in contrast to what is observed for any other amino acid, induces only very slight inhibition of the initiation of chromosome and minichromosome replication in a relaxed strain of *E. coli* although fully inhibiting their synthesis, and that of pBR322, in a stringent strain. Starvation for any amino acid inhibits the rate of RNA synthesis only in the stringent strain, as previously reported (Cashel, 1975; Gallant, 1979), whereas protein synthesis is fully inhibited in both stringent and relaxed strains after starvation for either isoleucine or arginine. From these results we conclude that initiation of *oriC* and pBR322 replication is stringently regulated and that isoleucine seems not to be essential for the protein synthesis required at the initiation of *oriC* replication.

2. Materials and methods

(i) *Bacterial strains and plasmids*

Bacterial strains used in this study were *Escherichia coli* LE234 which is K12 F⁻ *metB argH ilv thi*, BP225 is LE234 *relA*, FC200 is LE234 *ilv*⁺ and BP226 is FC200 *relA*. pBR322 was obtained from R. Diaz. pSY317 is a minichromosome containing a 5·6 kb *Eco* RI *E. coli oriC* fragment and a 7·9 kb kanamycin-resistance fragment, and was obtained from A. Kornberg (Kaguni, Fuller & Kornberg, 1982).

(ii) *Growth conditions*

Bacteria were grown in M9 minimal medium with appropriate supplements and growth was monitored by the absorbance at 450 nm. Overnight cultures were diluted 200 times in the same medium and treatments were begun in mid logarithmic phase. Inhibition of RNA synthesis was carried out by adding rifampicin (150 μg/ml). Protein synthesis was inhibited by chlor-

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amphenicol (200 μg/ml), or by removing the required amino acids by filtration. Deprivation of isoleucine was carried out by removing the required exogenous isoleucine to *ilv*⁻ strains or by depletion of endogenous isoleucine by valine addition (500 μg/ml) to *ilv*⁺ strains.

(iii) *Measurement of DNA, RNA, and protein synthesis*

DNA synthesis was measured by growing bacterial cells in minimal medium containing [³H]thymidine (Amersham) at a concentration of 1 μg/ml (37 kBq/μg) and 1·5 mM uridine, and measuring the TCA precipitable radiactive material. Minichromosome and plasmid replication were measured by the [³H]-thymidine incorporated into the plasmid band cut out from a 0·7% agarose gel electrophoresis of a crude lysate (Eckhardt, 1978). The rate of RNA synthesis was measured by the TCA precipitable radioactive material incorporated in 3 min in 1 ml aliquots of the culture containing 18·5 kBq/ml [³H]uridine (Amersham). Protein synthesis was measured by growing bacterial cells in the presence of [³H]arginine (New England Nuclear) or [³H]-methionine (Amersham), at a concentration of 40 μg/ml (4·6 kBq/μg), for at least four mass doublings prior any treatment, and measuring the incorporated isotope as TCA precipitable material.

3. Results

To study the effect of the stringent response upon DNA replication we measured minichromosome and pBR322 DNA synthesis in a stringent strain and in its relaxed counterpart after inhibiting protein synthesis by different treatments. As Fig. 1 shows, no replication of minichromosome pSY317 was observed after chloramphenicol addition or arginine or valine starvation, which indicates that initiation at *oriC* has a strict requirement for protein synthesis. The synthesis of pBR322 DNA does not have his requirement (Clewell & Helinski, 1972) and its inhibition by amino acid starvation in the stringent strain may be ascribed to the inhibitory effect of the activated synthesis of ppGpp (Hecker, Schroeter & Mach, 1983). When bacterial cells were starved for isoleucine by removing the required isoleucine in LE234 and BP225 or by adding valine to their *ilv*⁺ counterparts, both replicons were inhibited in the stringent strains but only very slight inhibitions were observed in the relaxed mutants (Fig. 1) even though the macromolecular synthesis of the culture, as detected by mass increase, ceased after isoleucine starvation. Growth was also inhibited by starving for any other amino acid, or by addition of chloramphenicol (data not shown).

We wanted to know if this observed difference was due to continued protein synthesis in the Rel⁻ strain after removing isoleucine caused by differences in the

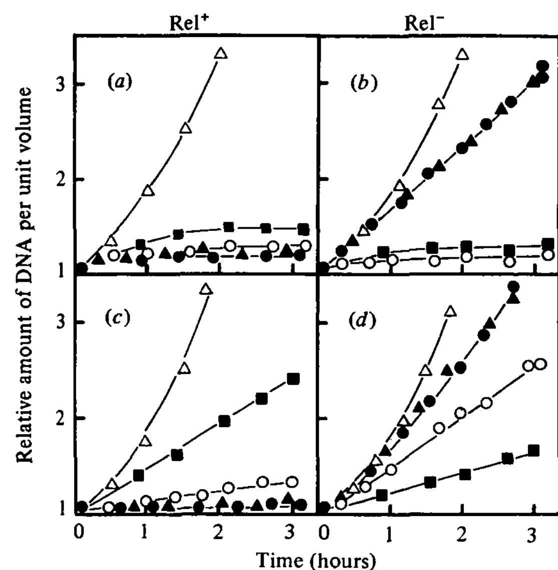

Fig. 1. Relative amount of pSY317 (*a*, *b*) and pBR322 (*c*, *d*) DNA synthesized in the Rel⁺ strains LE234 and FC200 (left panels) and the Rel⁻ strains BP225 and BP226 (right panels). The different treatments are indicated as follows: strains without any treatment (△); addition of chloramphenicol (■); FC200 or BP226 with added valine (▲); LE234 or BP225 starved for isoleucine (●); LE234, FC200, BP225 or BP226 starved for arginine, arginine and isoleucine, and LE234 or BP225 starved for valine and isoleucine (○).

pools of this amino acid in both strains. To measure protein synthesis bacterial strains were grown in the presence of [³H]arginine prior to starvation for isoleucine by removing this required amino acid to *ilv*⁻ strains or by addition of valine to *ilv*⁺ strains, or in the presence of [³H]methionine prior to starvation for isoleucine as above or the required arginine. Figure 2 shows that the starvation of isoleucine or arginine stops total protein synthesis in the Rel⁺ and in the Rel⁻ strains which suggests no detectable differences in pools.

The observed difference in the replication of the plasmids after amino-acid starvation could also be ascribed to inhibition of the uptake of radioactive thymidine due to an effect of the stringent response (Lin-Chao & Bremer, 1986). To study the effect of isoleucine deprivation on pBR322 replication in stringent and relaxed strains aside from exogenous thymidine, pBR322 containing Rel⁺ and Rel⁻ strains growing in minimal medium were starved for isoleucine by valine addition or treated with chloramphenicol, and agarose gel electrophoresis of crude lysates of 0·5 ml aliquots were performed after 10 h. Figure 3 shows that starvation for isoleucine inhibits replications only in the Rel⁺ strain (lane 2) and this very treatment produces amplification in the Rel⁻ strain (lane 4) with a better yield than with chloramphenicol treatment (lanes 1 and 3). From this result we can conclude that inhibition of replication by isoleucine deprivation in the Rel⁺ strain is not the

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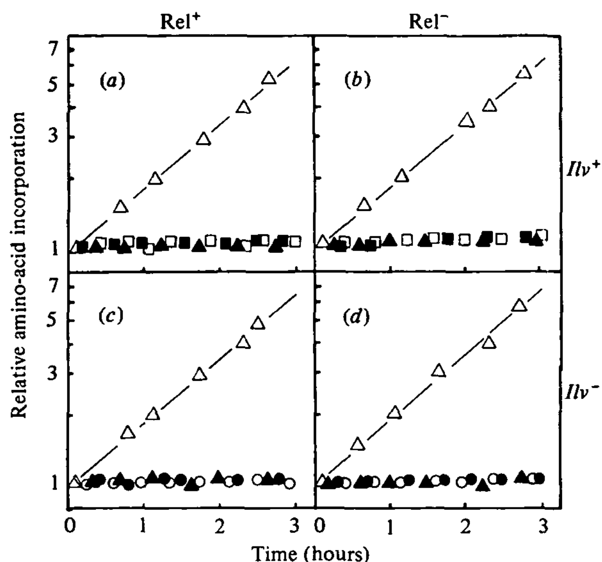

Fig. 2. Protein synthesis measured by [³H]arginine (open symbols) or [³H]methionine incorporation (closed symbols) in Rel⁺ (left panels) and Rel⁻ (right panels) strains exponentially growing (△) and after starvation for arginine (▲), addition of valine to FC200 (*a*) and BP226 (*b*) (■, □) or starvation for isoleucine to LE234 (*c*) and BP225 (*d*) (●, ○). Initial value for [³H]arginine is 8×10^4 cpm, and for [³H]methionine is 2×10^4 cpm.

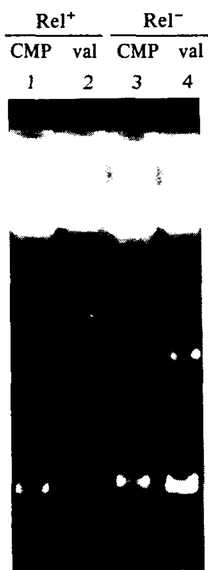

Fig. 3. Agarose gel electrophoresis of crude lysates of 0·5 ml aliquots of pBR322 containing FC200 (lanes 1 and 2) and BP226 (lanes 3 and 4) grown in minimal medium, 10 h after addition of chloramphenicol (lanes 1 and 3) or valine (lanes 2 and 4).

effect of a lower thymidine uptake by the stringent response.

To study the onset of the stringent response we measured the rate of RNA synthesis after every treatment. Figure 4 shows that starvation for isoleucine or arginine promotes the stringent response but chloramphenicol addition does not, as previously reported (Cashel, 1975; Gallant, 1979).

These results confirm that protein synthesis is

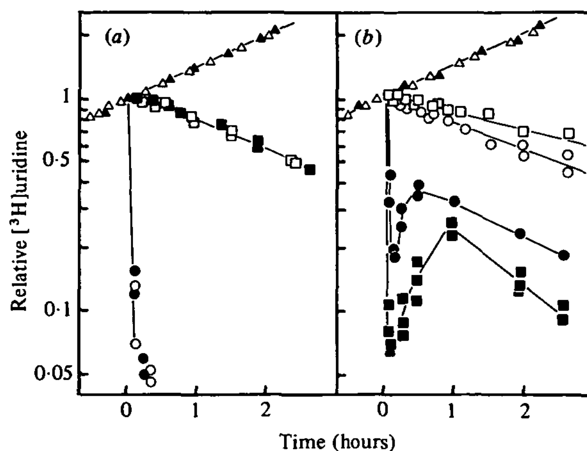

Fig. 4. Rate of RNA synthesis in LE234 (closed symbols) and BP225 (open symbols) in an exponentially growing culture (▲, △) and (*a*) after the addition of rifampicin (●, ○) or addition of chloramphenicol (■, □) and (*b*) after starvation for arginine (●, ○) or starvation for isoleucine (■, □).

required for *oriC* replication and, as isoleucine starvation inhibits protein synthesis in the stringent and relaxed strains to the same extent without inhibiting pSY317 replication in the relaxed strain, we conclude that isoleucine seems not to be essential for this synthesis.

The inhibition of pSY317 and pBR322 replication by isoleucine deprivation in the Rel⁺ strain suggest that the stringent response inhibits initiation of both replicons. Isoleucine starvation thus promotes amplification of both minichromosome and pBR322 in the Rel⁻ strain, the effect on pBR322 being greater than that of chloramphenicol treatment (Figs 1 and 3).

It was of interest to see if the stringent response and isoleucine starvation had the same effect on chromosome replication. As RNA and protein synthesis are only required at the initiation step of replication, the accumulation of DNA by the elongation activity of the replication forks after inhibiting those macromolecules is an estimate of the inhibition of that initiation step. Figure 5 shows that the inhibition of RNA or protein synthesis inhibits the initation step in any strain but only a slight inhibitory effect was observed after the starvation for isoleucine or addition of valine in the *rel* mutants. These results show that the effects of the stringent response and the starvation for isoleucine on the initiation of chromosome replication are similar to those observed in pSY317.

4. Discussion

We have studied the effect of the stringent response upon *oriC*-dependent DNA replication and on replication of pBR322. The results after starving of required amino acids show that the replication of *oriC* requires protein synthesis, because pSY317 replication is inhibited by amino-acid starvation in the Rel⁻ strain

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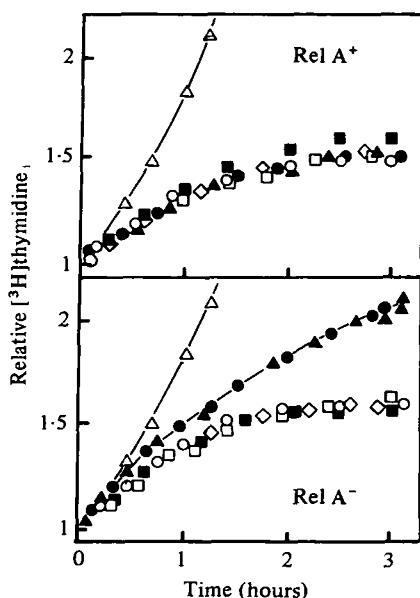

Fig. 5. Relative accumulation of DNA in LE234 and FC200 (upper panel) and in BP225 and BP226 (lower panel) growing exponentially (△) and after adding at 0 time chloramphenicol (■) or valine (▲) or after starvation for arginine (○) or isoleucine (●) or arginine and isoleucine (◇) or valine and isoleucine (□).

and by treatment with chloramphenicol in both strains. pBR322 replication does not require protein synthesis (Clewell & Helinski, 1972), and its inhibition by amino acid deprivation in the stringent strain may be ascribed to the stringent response (Hecker, Schroeter & Mach, 1983).

When protein synthesis was prevented by isoleucine starvation, either by removing the required exogenous isoleucine for ilv^- strains or by depletion of endogenous isoleucine by valine addition to ilv^+ strains, neither replicon showed inhibition of replication in the *rel* mutants. As protein synthesis is inhibited by arginine or isoleucine starvation to the same extent in Rel^+ and Rel^- strains, the effect of isoleucine starvation on replication can not be explained by different pools in stringent and relaxed strains. From this we conclude that either this amino acid is not essential for the protein synthesis required for initiation at *oriC* or the isoleucine content of the initiation protein(s) is so low as to permit its synthesis by re-utilization of isoleucine from protein degradation.

As isoleucine is required for protein synthesis, it follows that its deprivation can be used as an amplification method for these two plasmids. This method is even more convenient for pBR322 in a *relA* strain since chloramphenicol produces lower amplification factors.

Minichromosome and plasmid replication are inhibited by isoleucine starvation only in the Rel^+ strain. As this amino acid seems not to be required for the protein synthesis required for their replication, we conclude that both replicons are inhibited by the stringent response.

The experiments shown in Fig. 5 reveal that isoleucine seems not to be necessary for the protein synthesis required for the initiation of chromosome replication. The inhibition of chromosome replication by isoleucine starvation in the Rel^+ strain can be attributed to the onset of the stringent response.

As protein synthesis is required for the initiation of chromosome replication and as isoleucine is a common amino acid, these results suggest that most likely only one unstable protein could be required for the initiation step of chromosome replication, and that this protein does not contain isoleucine, although at present we can not rule out the synthesis of this protein(s) by re-utilization of isoleucine from protein degradation.

Recently Rokeach and Zyskind (Rokeach & Zyskind, 1986) have shown that cells starved for isoleucine, but not those treated with chloramphenicol, have greatly reduced levels of an RNA entering *oriC* that is presumed to have a role in initiating replication. This transcript is stringently regulated and it could explain the effect of the stringent response at the initiation of replication reported in this paper.

That ppGpp plays an inhibitory role at the initiation of replication in *oriC* and pBR322 replicons has also been confirmed by us. An exponentially growing *relA relX* double mutant, which has a ten-fold reduced basal level of ppGpp (Pao & Gallant, 1978), has a 70% increase in the chromosome and pSY317 copy number and a 170% increase in the pBR322 copy number per unit mass compared with its isogenic Rel^+ strain (data not shown). These results lead us to suggest that the level of ppGpp can affect the initiation frequency of chromosome and plasmid replication most likely by its inhibitory effect on the synthesis of the initiator RNA, although the results with chromosome and minichromosome could also be explained by stringent regulation of the mRNA for a protein required for initiation. In this sense it is interesting to mention the finding of an inverse correlation between cellular ppGpp concentration and growth rate (Ryals, Little & Bremer, 1982) which correlates with the finding that the lower the growth rate the higher the cellular concentration of ppGpp and the lower the chromosome initiation frequency. This suggestion points towards ppGpp as a putative chemical messenger in translation, transcription and replication.

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