THE INFLUENCE OF dna A AND dna C MUTATIONS ON THE INITIATION OF PLASMID DNA REPLICATION

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SUMMARY: The dependence of the replication of several plasmids on the chromosome-determined initiation products, dna A and dna C, has been studied. The initiation of the replication of Col E_1 DNA requires the chromosomal dna A product. In contrast two de-repressed transfer factors (R 1 drd 16 and Hly_{152}) seem to determine a corresponding plasmid-specific factor. The dna C-product is necessary for the ordered initation of all plasmids studied. The addition of low concentrations of chloramphenicol leads to a relaxed replication of Col E_1 DNA at the restrictive temperature in dna A-mutants, but not in dna C-mutants.

INTRODUCTION: The replication rate of the chromosome in E.coli is determined by the frequency of initiation (1,2). Protein synthesis is required for the initiation step. Evidence exists that at least two different proteins are involved in this process, the syntheses of which are inhibited differently by chloramphenicol (3,4). The isolation of two distinct classes of mutants of E.coli (dna A and dna C), which are thermosensitive in the initiation process (5), supports these data. However, there is no evidence that the thermosensitive products of these mutants are identical with the former proteins. In addition to these regulatory proteins a fixed origin on the DNA (6), RNA polymerase and RNA synthesis at or near the origin (4,7) are also prerequisites for the initiation of replication of the chromosomal DNA of E.coli. Several models with positively and negatively functioning control elements have been proposed to describe the regulation of this process (8-10). Studies on extrachromosomal DNA elements (plasmids) in E.coli have suggested that some factors, which are necessary for the replication of these DNA elements, are plasmid-specific, whereas others are determined by the chromosome (11-15). At least one of the products coded by plasmids seems to be involved in the initiation of replication of these plasmids (8,16,17).

There is evidence, which suggests that the replication of the two small plasmids, Col E_1 DNA and minicircular DNA of E.coli 15, differs considerably from that of the larger plasmids (14,18), which are mostly sex factors. The ability of the two small plasmids to replicate in the presence of chloramphenicol (19 and Goebel, W. and Schroen, W. manuscript in preparation) suggests, that the initiation of these plasmids does not require de novo protein synthesis. It therefore seemed likely that the initiation of Col E_1 and minicircular DNA may be different from that of the chromosome and the larger plasmids, which is dependent on newly synthesized protein(s). We report here the first results on studies on the replication of several plasmids in temperature-sensitive initiation mutants of E.coli (dna A and dna C) The results indicate that the mechanism of initiation may be different for the two types of plasmids.

MATERIALS AND METHODS: E.coli 120/6 (from Dr. Bonhoeffer), CRT 46 (from Dr. Hirota), PC 2 (from Dr. Carl) and dna C-807 (from Dr. Beyersmann) were used in these studies. Col E_1 DNA was transferred into these strains by a Hfr H (Col E_1) donor strain. The hemolytic plasmid Hly_{152} was introduced into the mutant strain by mating with E.coli K 12 (Hly_{152}). The donor strain for the de-repressed antibiotic-resistance factor R 1 drd 16 originates from E.coli W 945 (R 1 drd 16) which was kindly provided by Dr. E. E. Meynell. The isolation of plasmid DNA by the lysozyme-Brij 58 procedure followed by dye-buoyant densitiy centrifugation and the other experimental details are described in the legends to the figures.

RESULTS: We have reported previously (13) that Col E_1 DNA synthesis decreases rapidly in the temperature-sensitive initiation mutant Hfr 120/6 (Col E_1) (20) at the elevated temperature (fig. 1 A). The incorporation of ^{3}H-thymidine into the hemolytic DNA Hly_{152} and the antibiotic resistance factor R 1 drd 16, both of which are large, de-repressed transfer factors (21 and Goebel, W. and Pokora-Royer, B. manuscript in preparation), also declines at the restrictive temperature (42°C). The decrease follows roughly the reduction in the incorporation of ^{3}H-thymidine into chromosomal DNA (fig. 1 A). A similar observation has been recently described for the replication of F'lac DNA in an initiation mutant of S.typhimurium (22). Col E_1 DNA synthesis is restored in this mutant to a considerable extent at the restrictive

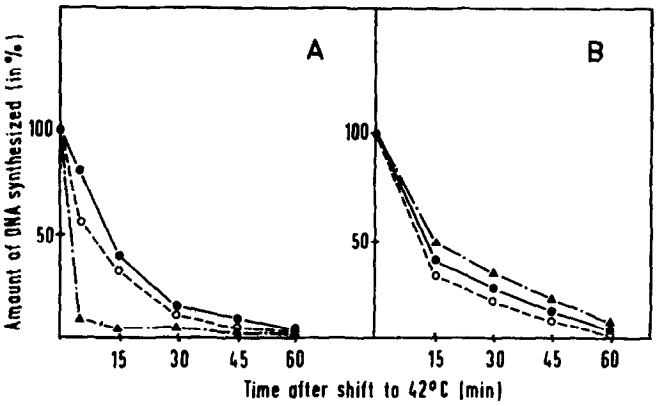

Fig. 1: Incorporation of ^{3}H-thymidine into chromosomal DNA and plasmid DNA in the mutant 120/6 at 42°C.
Cultures of E.coli 120/6 carrying either Col E_1, R 1 drd 16 or Hly_{152} or a combination of Col E_1 + Hly_{152} or R 1 drd 16 were labelled with 5 µCi/ml ^{3}H-thymidine (spec.act. 20,4 Ci/mmol) for 30 min. at the indicated time after shift to 42°C. (Cell density at the time of the shift was 3 x 10^{8} cells/ml). Phosphate-buffered minimal medium (13) supplemented with 10 µg/ml thymine was used for culturing the strains. Cleared lysates were prepared by the lysozyme-Brij 58 procedure (27). Supercoiled plasmid DNA was isolated from the cleared lysates by CsCl-EtBr gradient centrifugation (28) and subsequent separation of the supercoiled DNA on neutral sucrose gradients as described before (13). Alternatively, the cleared lysates were directly centrifuged on alkaline sucrose gradients (29).
Both methods gave essentially the same results. There was no indication of an appreciable amount of open circular plasmid DNA synthesized under these conditions. The percent values given are the amount of ^{3}H-radioactivity incorporated into supercoiled plasmid and chromosomal DNA at the indicated times at 42°C related to the amount of incorporation at 30°C at the time of the shift.
A. 120/6 (Col E_1) and 120/6 (R 1 drd 16)
B. 120/6 (Col E_1, R 1 drd 16)
•——• chrom. DNA, o --- o R 1 DNA, ▲—·—▲ Col E_1 DNA

temperature when one of the two large plasmids (Hly_{152} or R 1 drd 16) is present in the same cell (fig. 1 B), whereas the amount of the large plasmids synthesized at 42°C in the presence of Col E_1 DNA remains unchanged.

The temperature-sensitive mutation of the initiation mutant CRT 46, isolated by Hirota et al. (23) maps close to the ilv locus (dna A) (5). This mutant carries already a large extrachromosomal element of unknown function (80 S, supercoiled, unpublished results). The synthesis of Col E_1 DNA in this mutant (fig. 2 A) also declines at 42°C. However, the residual plasmid synthesis approaches the amount of Col E_1 DNA synthesized at 42°C in the mutant Hfr 120/6 when a large plasmid is

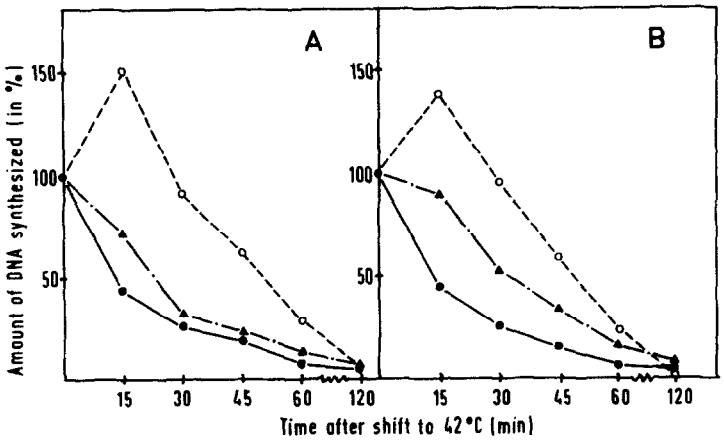

Fig. 2: Incorporation of ^{3}H-thymidine into chromosomal DNA and plasmid DNA in the mutant CRT 46, carrying Col E_1 or R 1 drd 16 and a combination of both plasmids.
Experimental conditions were the same as described in Fig. 1.
A. CRT 46 (Col E_1) and CRT 46 (R 1 drd 16)
B. CRT 46 (Col E_1, R 1 drd 16)
●——● chrom. DNA, o --- o R 1 DNA, ▲— —▲ Col E_1 DNA

present together with the Col E_1 factor. The incorporation of ^{3}H-thymidine into Hly_{152}-DNA and R 1 DNA is not inhibited during the first 20 minutes at 42°C. Then it decreases and stops about at the same time when the residual replication cycle of the chromosome is completed (fig. 2 A). A higher amount of Col E_1 DNA is again synthesized at 42°C when one of the two large plasmids (Hly_{152} od R 1) is carried by this mutant in addition to the Col E_1 factor. The increased amount of plasmid DNA synthesized in this mutant at 42°C as compared to mutant 120/6 may be due to a slower rate of denaturation of the dna A product. In addition, the large unknown plasmid of CRT 46 could support the synthesis of Col E_1 DNA in this mutant like the other two large plasmids

E.coli PC 2 is an initiation mutant, which has been isolated previously by Carl (24). Its temperature-sensitive mutation (dna C) is linked to the dra locus (5), which indicates that it is different from the dna A mutation of CRT 46 and possibly of Hfr 120/6. The other dna C mutant, E.coli dna C-807, used in these studies has been recently isolated and provided to us by Beyersmann (Beyersmann, personal communication). The incorporation of ^{3}H-thymidine into the described plasmids at the restrictive temperature in both mutants is quite different from that in the former dna A mutants. Incorporation of ^{3}H-thymidine into the large plasmids (Hly_{152} and R 1) stops almost immediately at 42°C (fig. 3). This indicates that these plasmids com-

plete the round of replication, in progress, but do not initiate further rounds at 42°C. In contrast, Col E_1 DNA synthesis decreases slowly at 42°C (fig. 3), which suggests that several new cycles of replication of this plasmid can be initiated at the restrictive temperature. The exact amount of Col E_1 DNA synthesized after 30 minutes at 42°C is difficult to determine, since a considerable amount of the Col E_1 DNA

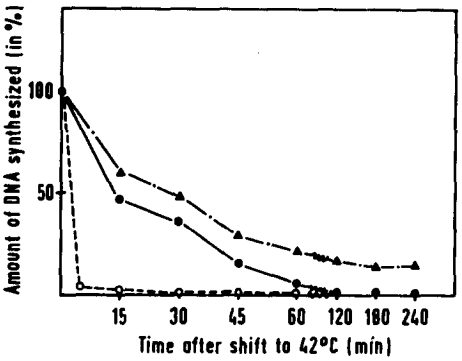

Fig. 3: Incorporation of ^{3}H-thymidine into chromosomal DNA and plasmid DNA in the mutant PC 2, carrying Col E_1 or R 1.
For experimental details see Fig. 1.
•— • chrom. DNA, o --- o R 1 DNA, ▲-·-▲ Col E_1 DNA

synthesized after this period consists of complex DNA molecules (oligomeric and catenated forms, to be published). Mainly low levels of these DNA forms are further synthesized when the round of replication of the chromosome is completed. Col E_1 DNA synthesis is not stimulated in the presence of either of the large plasmids (Hly_{152} or R 1), nor does the Col E_1 plasmid support the synthesis of the large plasmids at 42°C. The results obtained were identical in both dna C mutants (PC 2 and dna C-807).

When de novo protein synthesis of E.coli CRT 46 is inhibited at 42°C by chloramphenicol in a concentration of 150 µg/ml, the level of Col E_1 DNA synthesis increases considerably (fig. 4). This increase is even more pronounced, when only 3 µg/ml of chloramphenicol is added to the culture at 42°C (fig. 4 A). Under these conditions Col E_1 DNA synthesis is stimulated in the first 30 min. after the temperature shift and continues for more than 3 hr at 42°C at an almost uninhibited level. In contrast, the incorporation of ^{3}H-thymidine into the large plasmids (Hly_{152} and R 1) is slightly inhibited in the presence of 3 µg/ml chloramphenicol and almost completely with 150 µg/ml (fig. 4 B). The incorporation of radioactivity into chromosomal DNA seems to decrease

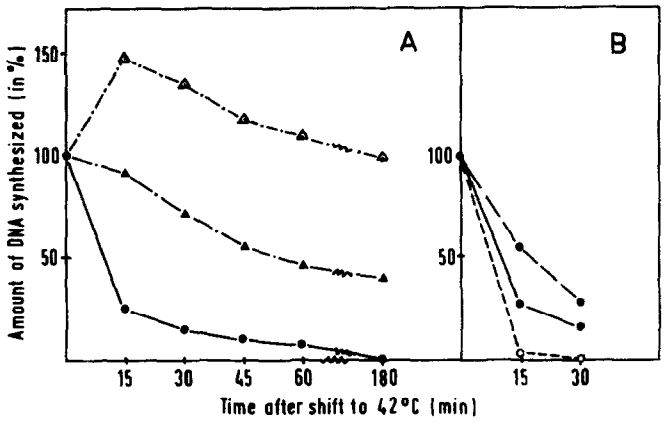

Fig. 4: Col E_1 and Hly_{152} DNA synthesis in the mutant CRT 46 at 42°C in the presence of chloramphenicol.
Cells of CRT 46 (Col E_1) or CRT 46 (Hly_{152}) were grown to a cell density of 3 x 10^8 cells/ml. The cultures were shifted to 42°C and chloramphenicol (CM) at a concentration of 150 µg/ml or 3 µg/ml was added at the time of the shift. Isolation of plasmid DNA was the same as described in Fig. 1.

A. CRT 46 (Col E_1):
- •—— • chrom. DNA (150 µg/ml CM)
- ▲—·—▲ Col E_1 DNA (150 µg/ml CM)
- △—·—△ Col E_1 DNA (3 µg/ml CM)

B. CRT 46 (Hly_{152}):
- •—— • Chrom. DNA (150 µg/ml CM)
- •– – –• Hly_{152} DNA (150 µg/ml CM)
- o– – –o Hly_{152} DNA (3 µg/ml CM)

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also in the presence of chloramphenicol at the restrictive temperature. The same effects by chloramphenicol are also observed at 42°C in the mutant 120/6 carrying Col E_1 or one of the large plasmids. This indicates further that the defect of these mutants may be identical.

In the two dna C mutants (PC 2 and dna C-807) chloramphenicol does not influence the residual synthesis of Col E_1 DNA at 42°C. There was also no stimulatory effect by chloramphenciol on the Col E_1 DNA synthesis in dna G-mutants (5) at the restrictive temperature. This product is involved in the elongation process of the chromosomal DNA replication, possibly by controlling the initiation of the discontinuosly synthesized DNA segments in the daughter strand (25). The dna G product is also required for Col E_1 DNA synthesis (unpublished results).

DISCUSSION: The strict dependence of Col E_1 DNA synthesis on the host-determined dna A-product suggests that this protein is directly involved in the initiation of replication of this plasmid. The transfer factors studied (Hly_{152} and R 1 drd 16) seem to determine a plasmid-specific "dna A-product", which may replace in part the chromosome-

determined dna A-product in the initiation of Col E_1 DNA. Further evidence for the existence of plasmid-specific "dna A-products" coded by Hly_{152} and R 1 drd 16 DNA is provided by the observation that both mutants, 120/6 and CRT 46, are suject to "integrative suppression" by these and other F-like plasmids (16, 17 and unpublished results). Col E_1 DNA synthesis is restored at the restrictive temperature in these mutants when chloramphenicol is added at the time of the temperature shift. This effect by chloramphenicol seems to be specific for dna A-mutants. It is not generally observed in DNA replication mutants, when the defective products are also required for the synthesis of the Col E_1 plasmid. This result can hardly be explained by assuming that the dna A-product is a positively acting "initiator" or part of such a product. It rather favors the interpretation that the Col E_1 plasmid regulates its DNA synthesis by synthesizing a repressor substance, the synthesis of which is extremely sensitive to chloramphenicol. The regulation of the initiation of the chromosome by a replication repressor has been previously proposed by Pritchard et al. and Rosenberg et al. (9, 10). The dna A-product could act as an "antirepressor" substance removing the repressor from the origin (10,26). A defective dna A-product would then lead to a permanently repressed state, whereas the stop of the protein synthesis would inhibit the synthesis of the repressor, leading to a de-repressed situation capable of further initiations.

The larger plasmids studied are not further replicated at 42°C in dna A-mutants, when the round of replication of the chromosome is completed. Inhibition of protein synthesis at 42°C results in a further decrease of the residual DNA replication of these plasmids. This suggests that another chromosome-determined protein must be synthesized for the initiation of replication of the large plasmids, the synthesis of which is dependent on an running replication cycle of the chromosome and sensitive to chloramphenicol. The dna C-product seems to be a possible candidate for this protein since both large plasmids are unable to further initiate new rounds of replication in the absence of a functioning dna C-product. If the dna C-product fulfills this requirement, the results would suggest that the Col E_1 plasmid, although it needs intact dna C-product, may reuse this product for new initiation cycles. In contrast, the larger plasmids, like the chromosome, would depend on the synthesis of new dna C-product for each new initiation cycle. The biochemical function of this initiation factor is unclear.

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