REPLICATION OF THE MINICIRCULAR DNA OF E.COLI 15 IS DEPENDENT ON DNA POLYMERASE I BUT INDEPENDENT OF DNA POLYMERASE III

Werner Goebel and Hildgund Schrempf

Institut für Mikrobiologie und Molekularbiologie, Universität Hohenheim, 7000 Stuttgart-Hohenheim, Germany

Received August 22,1972

SUMMARY: Minicircular DNA of E.coli 15 can be transferred together with Col E_1 DNA by a de-repressed sex factor into other strains of E.coli, which are pol^+. Both plasmids are lost, when the pol A 1 mutation is transferred into cells harbouring these plasmids. A mutant with a temperature-sensitive ploymerase I (pol A 12) is capable of replicating minicircular DNA and Col E_1 DNA at 30°C but not at 43°C. However, these latter plasmids are replicated at the restrictive temperature in a DNA replication mutant (dna E) with a temperature-sensitive DNA polymerase III.

Evidence has been recently presented that the colicinogenic factor E_1 (Col E_1) cannot be maintained in strains defective in DNA polymerase I (pol A 1) (1). Furthermore, experiments carried out in our laboratory have indicated that the maintenance and/or replication of Col E_1 DNA do not require the presence of polymerase III (2). In contrast, several sex factors (Col V, Col Ib, Hly, F'14, R1 and R 64) are replicated normally in pol A 1 mutants, but the replication of these factors requires DNA polymerase III (2). Minicircular DNA of E.coli 15 (3) and several other small plasmids isolated from various wild strains of E.coli have been shown to be closely related to Col E_1 DNA in their nucleotide sequences (4). In addition, minicircular DNA of E.coli 15 exhibits some physiological properties in common with Col E_1 DNA e.g., the replication of both plasmids proceeds in the presence of chloramphenicol (5,6) and at the restrictive temperature in a class of temperature-sensitive DNA replication mutants (dna B) (7). Both plasmids are present in the bacterial cell in several copies, which are selected randomly for replication (7,8).

It was therefore of interest to examine whether minicircular DNA of E.coli 15 would also show the same unusual requirement for DNA polymerase I as Col E_1 for its maintenance and/or replication. We have therefore examined

the ability of the minicircular DNA of E.coli 15 to replicate in the absence of the DNA polymerases I or III. The results suggest that DNA polymerase III is not required for the replication of the minicircular DNA of E.coli 15, but the replication and maintenance of this bacterial plasmid is strongly dependent on a functioning DNA polymerase I.

MATERIALS AND METHODS: The bacterial strains used in this study (listed in Table 1) were grown in phosphate-buffered minimal medium (6). Isolation

Table 1: BACTERIAL STRAINS and their properties

Strain	Properties	Source
K 12 ($Hly_{PM\ 152}$)	lac^-, nal^r	H.W.Smith
15 THU^- (Col E_1, $mini_{15}$)	thy^-, his^-, ura^-, str^r, Col E_1, $mini_{15}$	6
W 3110 pol A 1	thy^-, pol A 1	20
MM 383	thy^-, su^-, rha^-, str^r lac^-, pol A 12	15
1026	str^r, dna E	17

of the supercoiled plasmid DNA was performed by the lysozyme-Brij 58 technique (9).

Transfer of the de-repressed Hly-factor, originated from E.coli PM 152 (10), to E.coli 15 THU^- (Col E_1, $mini_{15}$) was performed by mating E.coli 15 THU^- (Col E_1, $mini_{15}$) with E.coli K 12 ($Hly_{PM\ 152}$) (10). The conjugal transfer of the Col E_1 factor and the minicircular plasmid which both lack sex factor activity, was promoted by this Hly factor. Log phase cultures of the donor strain E.coli 15 THU^- (Hly, Col E_1, $mini_{15}$) and of the desired recipient strain were mixed on ENB-agar plates (6) and incubated overnight. The recipient was isolated on appropriate media. The resulting colonies

were examined for the presence of the Col E_1 factor by testing for colicin production as described by Frédéricq (11). The pol A 1 mutation was transferred to E.coli K 12 met E (Col E_1, $mini_{15}$) by conjugation with E.coli W 3110 pol A 1 (Hly). Methionine prototrophic colonies were counter selected on an appropriate minimal medium lacking methionine. These colonies were further examined for sensitivity to methyl methanesulfonate (MMS). MMS-sensitive colonies were cultured in ENB broth. Crude extracts of these cells were prepared and DNA polymerase I activity was assayed as described by Kohiyama and Kolber (12).

Centrifugation conditions and other experimental details are given in the legends to the figures.

RESULTS and DISCUSSION:

1. Transfer of the minicircular DNA of E.coli 15

The minicircular DNA of E.coli 15 ($mini_{15}$) is a small plasmid with unknown function, which does not possess transfer properties (3,13). It is therefore difficult to transfer this extrachromosomal element to other bacterial strains. The Col E_1 factor, which determines the production of the antibiotic protein colicin E_1 closely resembles the minicircular DNA of E.coli 15 in its nucleotide sequences (4), and seems to have (a) replication site(s) in common with this latter plasmid (6). The Col E_1 plasmid also cannot promote its own transfer. However it appeared reasonable to assume, that both plasmids may be co-transferred by an appropriate transfer factor into other strains of E.coli. Recently, we have isolated mutants of E.coli 15 (Col_1), which are cured for the large P_1-like plasmid (6), inherent to all strains of E.coli 15 like the minicircular DNA (14). We have transferred to a cured mutant a de-repressed α-hemolytic factor, which exhibits a high efficiency of transfer with all strains of E.coli tested (10). With the use of this donor, the minicircular plasmid of E.coli 15 and Col E_1 DNA are co-transferred into E.coli K 12 strains with a frequency of 1 - 5 %. The amount of the minicircular DNA and Col E_1 DNA is about 1 % of the

total cellular DNA in all recipients which we obtained so far. However, the ratio between these two small plasmids varies from recipient to recipient and depends on the growth temperature. Using this donor strain, *E.coli* 15 (*Hly*, *Col* E_1, $mini_{15}$) we have transferred the minicircular plasmid together with the *Col* E_1 factor, a) to the *E.coli* K 12 wild strain, b) to a *pol* A 12 mutant (MM 383), possessing a temperature-sensitive polymerase I (15), and c) to a dna E mutant (1026), possessing a temperature-sensitive polymerase III (16,17).

2. Stability of the minicircular plasmid in *pol* A^+ and *pol* A 1 strains of *E.coli* K 12.

A colicinogenic non-hemolytic recipient strain of *E.coli* K 12 *pol* A^+ obtained by mating *E.coli* K 12 with *E.coli* 15 THU^- (*Hly*, *Col* E_1, $mini_{15}$) as described above, was cultured in 30 ml of phosphate-buffered minimal medium. ^{3}H-thymidine (5 μCi/ml) was added to the cells in the logarithmic growth phase. The plasmid DNA was isolated by the lysozyme-Brij 58 technique (9). The resultant "cleared lysate" was subsequently centrifuged to equilibrium in a cesium chloride-ethidium bromide (CsCl-EB) gradient (18). This procedure results in the separation of the supercoiled plasmid DNA, which bands in the gradient at a higher density, from the residual chromosomal and relaxed plasmid DNA, which band at a lower density (18). The fractions, containing supercoiled DNA were pooled and further analyzed on a neutral linear sucrose gradient (Fig. 1A). Supercoiled *Col* E_1 DNA (23 S) and minicircular DNA (16 - 17 S) are not quite separated under these conditions. The separation of the latter two plasmids can only be performed on an alkaline sucrose gradient, since the open circular form of *Col* E_1 DNA (17 S) overlaps under neutral centrifugation conditions with the supercoiled form of the minicircular DNA. The fractions of the neutral gradient between 16 - 23 S were therefore re-centrifuged on an alkaline gradient. Fig. 1B demonstrates the presence of supercoiled *Col* E_1 DNA at 48 S and of supercoiled minicircular DNA at 30 - 32 S under

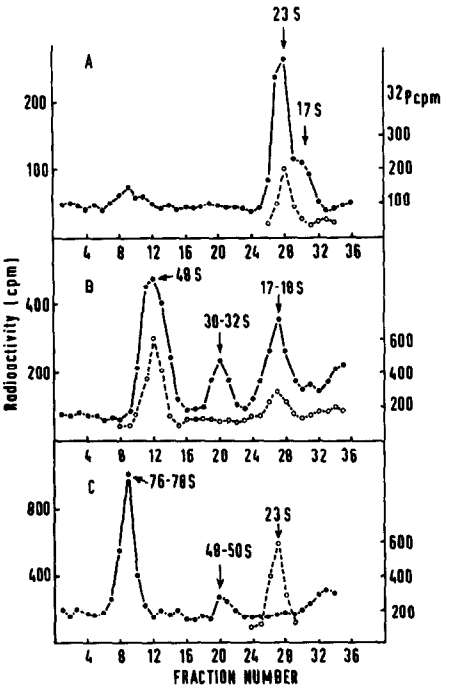

Fig. 1: Sucrose gradient analysis of the supercoiled plasmid DNA of *E.coli* K 12 (*Hly*, *Col* E_1, *mini*$_{15}$) and its corresponding *pol* A 1 mutant. Cultures of *E.coli* K 12 (*Hly*, *Col* E_1, *mini*$_{15}$, *pol* A^+) and its corresponding *pol* A 1 mutant were grown at 37°C and labelled at a cell density of 5 x 10^8 cells/ml with ^{3}H-thymidine (5 µCi/ml) for 1 h in the presence of 250 µg/ml deoxyadenosine. Cleared lysates of both cultures were prepared and centrifuged to equilibrium in CsCl-ethidiumbromide gradients. Fractions of the heavy density regions containing the supercoiled plasmid DNA were pooled, dialyzed and centrifuged through neutral gradients (45000 rev./min, 60 min, 20°C, SW 65 rotor, L2-50B centrifuge) Fractions (10 drops) were collected from the bottom of the tube in small vials, from which samples (10 µl) were taken for the determination of the radioactivity. For the separation of *Col* E_1 DNA and minicircular DNA the corresponding fractions (26 - 31) of the neutral sucrose gradient were re-centrifuged on an alkaline 5% to 20% sucrose gradient, containing 0,7 M NaCl and 0,2 M NaOH (45000 rev./min, 90 min, 20°C, SW 65, L2-50B). Fractions (10 drops) were collected from the bottom of the tube directly on filter papers, which were washed, dried, and counted in a liquid scintillation counter (SL 30 Intertechnique/France).
(A) - Neutral sucrose gradient of the supercoiled DNA of *E.coli* K 12 (*Hly*, *Col* E_1, *mini*$_{15}$, *pol* A^+).
(B) - Alkaline sucrose gradient of fractions 26 - 31 of the gradient A.
(C) - Neutral sucrose gradient of the supercoiled DNA of the *pol* A 1 mutant.
----- ^{32}P-labelled *Col* E_1 DNA used as internal marker.

these conditions. The slower sedimenting DNA species (17 - 18 S) represent the open circular form of Col E_1 DNA.

A met E mutant of this E.coli K 12 strain was selected and crossed with E.coli W 3110 pol A 1 (Hly).The two genes met E and pol A 1 are close together on the chromosme of E.coli and are co-transferred with a relatively high frequency (19). E.coli K 12 met^+-colonies were isolated and further examined for sensitivity to methyl methanesulfonate (MMS). Pol A 1 strains are known to be sensitive to MMS (19). Among the met^+-isolates tested two colonies could be obtained, which were sensitive to MMS. These two colonies were also non-colicinogenic. Polymerase I activity could not be detected in extracts of these strains. Plasmid DNA was isolated as described above. The results are shown in Fig. 1C. It is evident that neither Col E_1 DNA nor minicircular DNA is present in the pol A 1 strain, whereas Hly DNA is observed sedimenting at 76 - 78 S as supercoiled DNA, and at 48 - 50 S as open circular DNA.

3. Failure of the minicircular DNA and Col E_1 DNA to replicate at 43°C in the mutant MM 383.

MM 383 is a mutant with a temperature-sensitive DNA polymerase I. This mutant has been recently isolated by Monk and Kinross (15). The mutation is not identical to the pol A 1 mutation previously isolated by DeLucia and Cairns (20), and has been therefore designated pol A 12. We have transferred Col E_1 DNA with E.coli HfrH (Col E_1), and Col E_1 DNA together with minicircular DNA with E.coli 15 (Hly, Col E_1, $mini_{15}$) as donor strains to this mutant. It proved to be difficult to obtain stable colicinogenic colonies of this mutant with either of the two donor strains. However, we finally succeeded in isolating the desired recipients. Fig. 2A and B indicate that a strain, which has been made colicinogenic by mating with E.coli 15 THU^- (Hly, Col E_1, $mini_{15}$), contains all three plasmids at 30°C. However when the temperature of a culture of this strain is raised to 43°C and ^{3}H-thymidine is added at this temperature, no incorporation

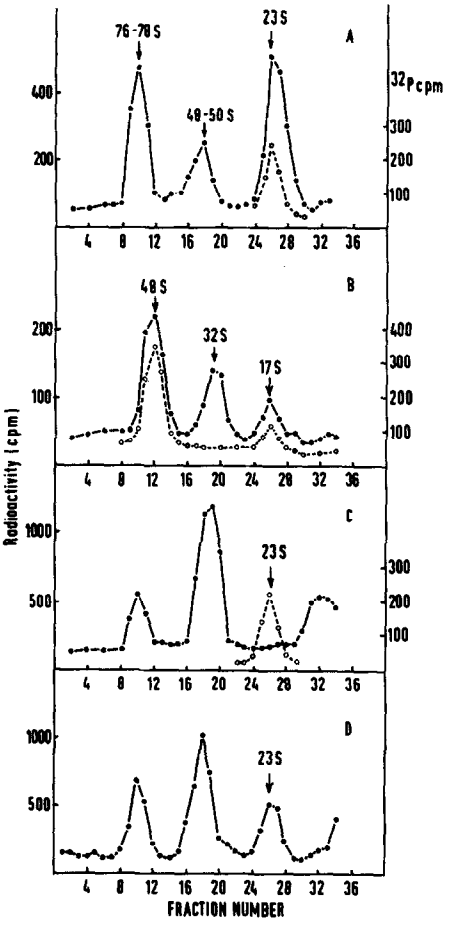

Fig. 2: Incorporation of ^{3}H-thymidine into plasmid DNA of the pol A 12 mutant MM 383 (Hly, Col E_1, mini$_{15}$) at 30°C and 43°C.
A culture of E.coli MM 383 (Hly, Col E_1, mini$_{15}$) was grown to the logarithmic phase (5 x 10^8 cells/ml) at 30°C and divided into three equal parts. One part was further incubated at 30°C and labelled with ^{3}H-thymidine (5 µCi/ml) for 1 h. The second part was incubated at 43°C and labelled with ^{3}H-thymidine (5 µCi/ml) for 1 h. The third part was incubated for 1 h at 43°C and labelled for 1 h with the same amount of ^{3}H-thymidine and then shifted back to 30°C and incubated for another hour. Supercoiled plasmid DNA was isolated as described in Fig. 1, and further analyzed on a linear sucrose gradient (5% to 20%). Centrifugation conditions were the same as described in Fig. 1.
(A) - Neutral sucrose gradient of the supercoiled plasmid DNA synthesized at 30°C.
(B) - Alkaline sucrose gradient of the fractions 25 - 29 of the gradient A.
(C) - Neutral sucrose gradient of the supercoiled plasmid DNA synthesized at 43°C.
(D) - Neutral sucrose gradient of the supercoiled plasmid DNA synthesized at 30°C after 1 h at 43°C.
----- ^{32}P-labelled Col E_1 DNA.

TABLE 2

Incorporation of C^{14}-Isoluecine, Valine and Phenylalanine into Protein by Isolated Liver Parenchymal Cells from Normal and Diabetic Rats*

(cpm/mg protein)

10mM Substrate	Normal Fed Rats	Diabetic Rats Hours after Insulin Withdrawal 0 hr	48 hr	72 hr	96 hr
Isoluecine	869 ± 58 (5)	1477±124 (5)	650 ± 86 (6)	463 ± 27 (3)	286 ± 57 (5)
Valine	1009± 85 (5)	1621±126 (5)	1176±118 (6)	528 ± 43 (3)	322 ± 50 (4)
Phenylalanine	764 ± 65 (5)	1206±119 (5)	1047±113 (6)	435 ± 85 (3)	200 ± 20 (4)

*A 1 ml aliquot of the Umbreit Ringer cell suspension was incubated for 1 hr in 2 ml of Umbreit Ringer buffer containing 10mM cold amino acid, 0.5 uCi of UL C^{14}-amino acid and 100 mg/100 ml glucose.

in diabetic rats deprived of insulin for 72 or 96 hr. Control cyclic AMP levels show a maximal level at 72 and 96 hr after the withdrawal of insulin. Similar increases in cyclic AMP levels in livers from acute alloxan diabetic rats have been reported by Jefferson et al. (11). They also found that livers from untreated alloxan diabetic rats have higher cyclic AMP levels than do livers from treated rats.

The decrease in the ability of glucagon to increase cyclic AMP levels or the decrease in C^{14}-amino acid incorporation into protein in isolated diabetic liver cells after the withdrawal of insulin is not a function of cell deterioration or changes in cell counts. While the cyclic AMP response to glucagon and rates of protein synthesis deminish, rates of gluconeogenesis are elevated. Gluconeo-

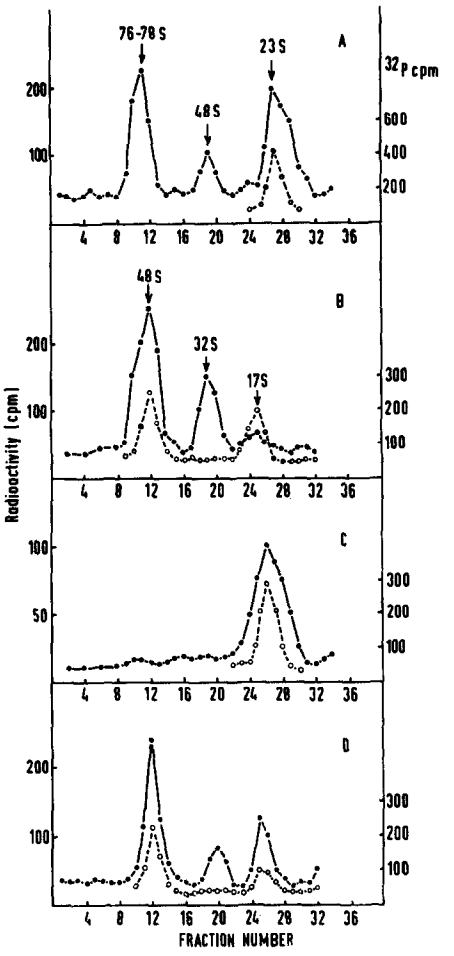

Fig. 3: Incorporation of ^{3}H-thymidine by plasmid DNA of the dna mutant 1026 (Hly, Col E_1, $mini_{15}$) at 30°C and at 45°C.
A culture of E.coli 1026 (Hly, Col E_1, $mini_{15}$) was grown to a cell density of 5 x 10^8 cells/ml at 30°C and then divided into two parts. One part was incubated further at 30°C and labelled with ^{3}H-thymidine (5 µCi/ml) for 30 min. The other part was shifted to 45°C and labelled at this temperature with 5 µCi/ml ^{3}H-thymidine for 30 min. Incorporation at 45°C was stopped by pouring the culture on a mixture of ice and water, containing 200 ug/ml sodium azide. Supercoiled plasmid DNA was isolated and analyzed on sucrose gradients as described in Fig. 1. Supercoiled Col E_1 DNA and minicircular DNA were again separated on an alkaline sucrose gradient.
(A) - Neutral sucrose gradient of the plasmid DNA of E.coli 1026 (Hly, Col E_1, $mini_{15}$) synthesized at 30°C.
(B) - Alkaline Sucrose gradient of the fractions 26 - 31of the gradient A.
(C) - Neutral sucrose gradient of the plasmid DNA synthesized at 43°C.
(D) - Alkaline sucrose gradient of the fractions 24 - 29 of the gradient C.
----- ^{32}P-labelled Col E_1 DNA.

in dna E mutants represents semi-conservative replication and not repair. It therefore seems reasonable to conclude that minicircular DNA is also replicated semi-conservatively in the absence of a functioning DNA polymerase III.

The data suggest, that there may be a class of related plasmids in E.coli which requires a DNA polymerase for its replication other than the chromosomal DNA and the larger plasmids known as sex-factors. The latter replicons seem to depend on DNA polymerase III for replication and probably require DNA polymerase I for repair. In contrast, Col E_1 DNA and minicircular DNA of E.coli 15, representing a possibly greater class of related plasmids, seem to require DNA polymerase I for replication and maintenance, whereas DNA polymerase III does not seem to be involved in these processes.

ACKNOWLEDGEMENTS: The authors wish to thank Prof. Dr. Lingens for his interest and support of this work. We are obliged to M. Monk for providing us the strain MM 383. Mrs. Mairi Sauer is thanked for reading of the manuscript. The skilful technical assistance of Mr. K. Kapassakalis is ackowledged.
This work was supported by a grant from the Deutsche Forschungsgemeinschaft.

1. Kingsbury, D.T. and D.R. Helinski, Biochem.Biophys.Rec.Comm., 41, 1538 (1970)
2. Goebel, W., Nature, 237, 67 (1972)
3. Cozzarelli, N.R., R.B. Kelly and A. Kornberg, Proc.Natl.Acad.Sci.US., 60, 992 (1968)
4. Goebel, W. and H. Schrempf, J.Bacteriol., in press
5. Clewell, D.B., J.Bacteriol., 110, 667 (1972)
6. Goebel, W. and W. Schroen, manuscript in preparation
7. Goebel, W. and H. Schrempf, Biochim.Biophys.Acta, 262, 32 (1972)
8. Bazaral, M. and D.R. Helinski, Biochemistry, 9, 399 (1970)
9. Clewell, D.B. and D.R. Helinski, Proc.Natl.Acad.Sci.US., 62, 1159 (1969)
10. Goebel, W. and B. Pokora, manuscript in preparation
11. Frédéricq, P., Ann.Rev.Microbiol., 11, 7 (1957)
12. Kohiyama, M. and A.R. Kolber, Nature, 228, 1157 (1970)
13. Lee, C.S. and N. Davidson, Biochim.Biophys.Acta, 304, 285 (1970)
14. Ikeda, H., M. Inuzuka and J. Tomizawa, J.Mol.Biol., 50, 457 (1970)
15. Monk, M. and J. Kinross, J.Bacteriol., 109, 971 (1972)
16. Gefter, M.L., Y. Hirota, T. Kornberg, J.A. Wechsler and C. Barnoux, Proc.Natl.Acad.Sci.US., 68, 3150 (1971)
17. Nüsslein, V., B. Otto, F. Bonhoeffer and H. Schaller, Nature, 234, 285 (1971)
18. Radloff, R., W. Bauer and J. Vinograd, Proc.Natl.Acad.Sci.US., 57, 1514 (1967)
19. Gross, J. and M. Gross, Nature, 224, 1166 (1969)
20. DeLucia, P. and J. Cairns, Nature, 224, 1164 (1969)