INCORPORATION OF DEOXYRIBONUCLEOSIDE TRIPHOSPHATES INTO PLASMID DNA BY TOLUENIZED CELLS OF THERMOSENSITIVE DNA REPLICATION MUTANTS OF *ESCHERICHIA COLI*

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1. Introduction

Since the isolation of a mutant of *E. coli* (W3110 Pol A1) with low DNA polymerase I (Kornberg DNA polymerase) activity by De Lucia and Cairns [1] it is generally assumed that this enzyme is not the true DNA replicase but may be required for DNA repair [2]. In addition, several classes of *E. coli* mutants which are temperature sensitive for DNA replication have been isolated, none of which possesses a temperature sensitive DNA polymerase I activity [3–5].

Recently, several systems have been described which are able to replicate DNA semiconservatively in vitro [6–8]. By using the toluenized bacteria system Kohiyama and Kolber [9] and Mordoh, Hirota and Jacob [10] have shown that temperature sensitive DNA replication mutants, in which the synthesis of DNA is immediately halted on shift from 30 to 42° (Dna B^--mutants), have an altered DNA replicase system, whereas the DNA polymerase I activity remains normal.

We have recently shown that the bacterial plasmid colicinogenic factor E_1 (Col E_1) is replicated to some extent in Dna B^--mutants at the elevated temperature [11, 12]. In this communication it is shown that deoxyribonucleoside triphosphates are incorporated at the elevated temperature into Col E_1 DNA by toluenized cells of Dna B^--mutants harboring the colicinogenic factor E_1. DNA polymerase I appears to be responsible for this incorporation.

2. Materials and methods

E. coli CR 34 Dna 43 and *E. coli* CR 34 Dna 43 (Col E_1) have been described previously [11]. *E. coli* CRT 2667 was a gift from Dr. Hirota. This strain was made colicinogenic for Col E_1 as described [13].

Treatment of bacteria with toluene and the assay for the DNA replicase system and for DNA polymerase I were performed essentially as described by Kohiyama and Kolber [9]. Modifications and other experimental details are given in the legends to figures.

3. Results and discussion

Incorporation of the four deoxynucleoside triphosphates (dNTP) at 30° by toluene-treated cells of Dna B^--mutant CR 34 Dna 43 (Col E_1) results in the synthesis of DNA, which sediments in a sucrose gradient with rather heterogeneous distribution (fig. 1). CsCl equilibrium centrifugation in the presence of the dye ethidium bromide [14] yields in addition to the chromosomal band a satellite band which is attributed to the supercoiled Col E_1 DNA (fig. 2). When ATP is omitted from the reaction mixture a sharp decrease of incorporation of dNTP is observed (table 1). Addition of *p*-hydroxymercuribenzoate, which is known to be a potent inhibitor for DNA replicase [7], leads also to a reduction of the incorporation of dNTP at 30° (table 1).

When the reaction is carried out at 42° and the lysate is centrifuged through a sucrose gradient, the high molecular weight DNA synthesized at 30°, is

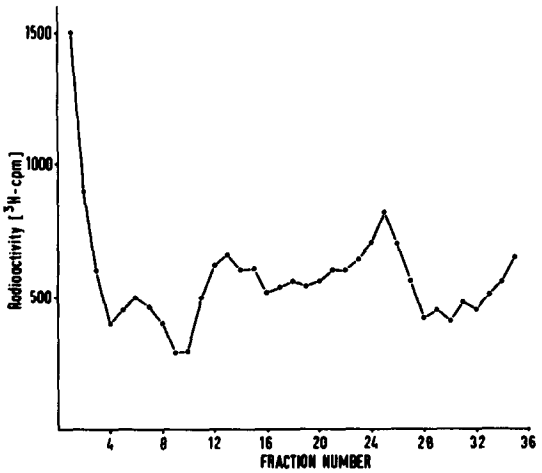

Fig. 1. Sedimentation analysis of DNA synthesized in toluene-treated cells of *E. coli* CR 34 Dna 43 (Col E_1) at 30°. Reaction mixture contained in a total of 0.15 ml: 0.05 M tris buffer (pH 7.3), 0.005 M $MgCl_2$, 0.09 M KCl, 3.5×10^{-5} M dNTP, 0.001 M ATP and 10 μCi/ml ^{3}H-TTP. 0.05 ml of toluenized bacteria (5×10^8 cells) was added to the reaction mixture. The reaction was terminated after 30 min at 30° by the addition of 0.1 ml of 0.1 M EDTA. 0.05 ml of 1% sodium dodecylsulfate (SDS) was added. The viscous lysate was layered after shearing through a pipette directly on a 5–20% neutral sucrose gradient and centrifuged for 120 min at 45,000 rpm in a Spinco type SW 65 rotor at 20°. 10-drop fractions were collected from the bottom of the tube on Selection filters GF 92 (Schleicher and Schüll, Germany). Each filter was successively washed with 10 ml of cold trichloroacetic acid (10%, w/v), 10 ml of hot water, 10 ml of ethylalcohol and 10 ml of ether. The filters were dried and the radioactivity was measured in a liquid scintillation counter (SL 40 Intertechnique).

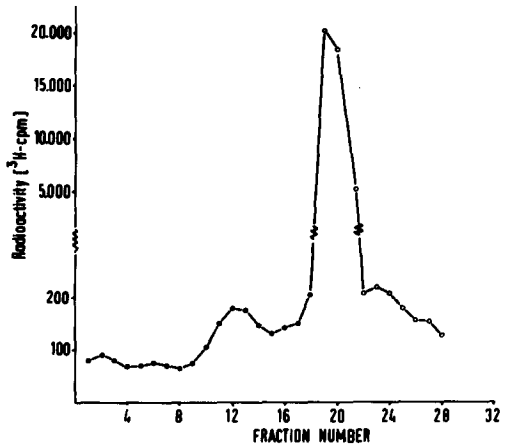

Fig. 2. Dye-buoyant density centrifugation of the DNA synthesized in toluene-treated cells of *E. coli* CR 34 Dna 43 (Col E_1) at 30°. The SDS-lysates of several reaction mixtures as described in fig. 1 were combined and sedimented in neutral CsCl in the presence of 125 μg/ml ethidium bromide for 18 hr at 44,000 rpm in a Spinco type 50 rotor at 2°. 15-drop fractions were collected on filter papers. The filters were washed, dried and counted as described in fig. 1.

absent, but two distinct DNA peaks, sedimenting at 23 S and 17 S, are still observed (fig. 3). This DNA probably represents Col E_1 DNA for the following reasons. i) dNTPs are not incorporated into these DNA components by toluene-treated noncolicinogenic cells of CR 34 Dna 43 (fig. 3). ii) The faster sedimenting DNA component cosediments with authentic supercoiled Col E_1 DNA which was added as marker in neutral and alkaline sucrose gradients (figs. 3 and 4). The sedimentation rate of the 17 S DNA remains practically unchanged in neutral and alkaline gradients. We therefore conclude that the DNA sedimenting at 23 S is monomeric supercoiled Col E_1 DNA, and the DNA, which sediments at 17 S, represents the corresponding open circular form. This Col E_1 DNA is obviously synthesized from deoxyribonucleoside triphosphate precursors at 42°.

Table 1
Incorporation of dNTP into DNA at 30° and 42° under various conditions.

Additions	DNA synthesized at 30°	DNA synthesized at 43°	
		Col E_1	Small-size DNA fragments
Complete system + tol. bacteria of *E. coli* CR 34 Dna 43 (Col E_1)	100%	100%	100%
- ATP	10%	100%	100%
+ *p*-hydroxymercuribenzoate (6×10^{-5} M)	15%	90–95%	90%
+ DNase (pancreatic) (0.3 μg/ml)	n.t.	10%	120%
Complete system + tol. bacteria of CRT 2667 (Col E_1)	95%	40%	35–40%

The reaction was the same as described in fig. 1. Incubation time was 40 min. For all assays 5×10^8 toluenized bacteria were used. n.t. = not tested.

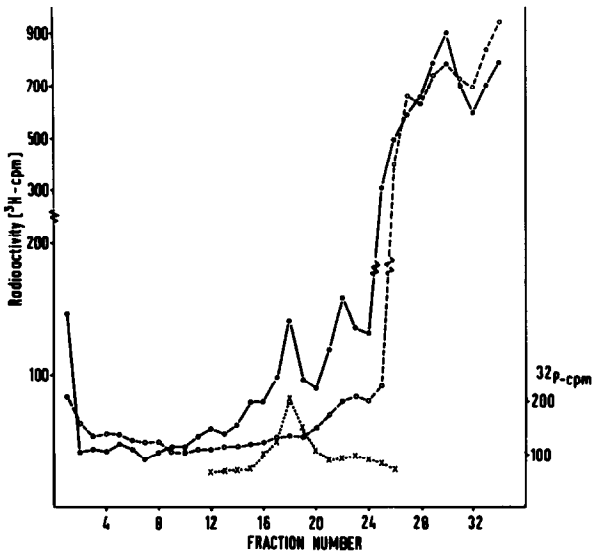

Fig. 3. Sedimentation analysis of DNA synthesized in toluenized cells of *E. coli* CR 34 Dna 43 (Col E_1) and *E. coli* Cr 34 Dna 43 at 42°. The reaction mixture and the experimental details were the same as described in fig. 1. The reaction was carried out at 42° for 40 min. ^{32}P-Labelled supercoiled Col E_1 DNA was added to the SDS-lysate, before layering it on top of the gradient. •——• DNA synthesized in toluenized cells of CR 34 Dna 43 (Col E_1); o– – –o DNA synthesized in toluenized cells of CR 34 Dna 43; x · · · x ^{32}P-labeled Col E_1 DNA (supercoiled).

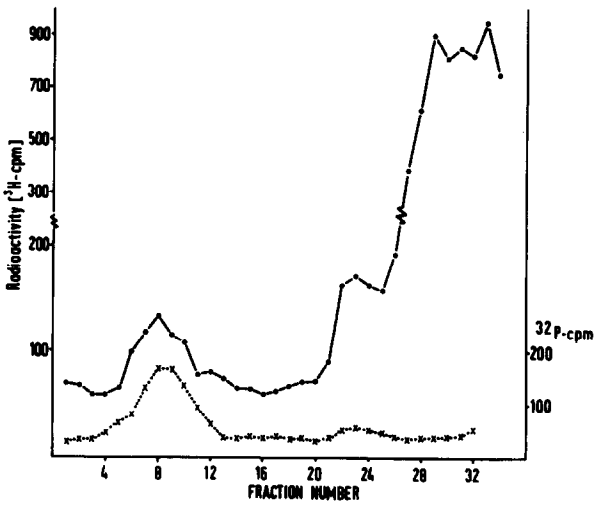

Fig. 4. Alkaline sedimentation analysis of DNA synthesized in toluene-treated cells of CR 34 Dna 43 (Col E_1) at 42°. DNA was synthesized in the reaction mixture described in fig. 1 at 42°. The SDS-lysate together with ^{32}P-labeled Col E_1 DNA was placed on a 5–20% alkaline sucrose gradient (0.7 M NaCl, 0.2 M NaOH, 1 mM EDTA) and centrifuged for 2 hr at 45,000 rpm in a SW 65 type rotor at 20°. 10-drop fractions were collected from the bottom of the tube on filter papers, which were washed, dried and counted as described. •——• DNA synthesized in toluene-treated cells of CR 34 Dna 43 (Col E_1); x · · · x ^{32}P-labeled Col E_1 DNA.

In contrast to the incorporation of dNTP into DNA at 30° the incorporation of dNTP into Col E_1 DNA at 42° is only slightly inhibited by *p*-hydroxymercuribenzoate at a concentration of 6×10^{-5} M (table 1). ATP does not seem to be required for the incorporation (table 1). Toluenized cells of a colicinogenic Dna B^--mutant, which carries in addition the Pol A 1 mutation [CRT 2667 (Col E_1)] [1, 10] incorporates dNTP into Col E_1 DNA at an evidently reduced rate (table 1) as compared to CR 34 Dna 43 (Col E_1) which is Pol A^+. The DNA polymerase I activity of a crude extract of this mutant was determined to be 5–10% of a Pol A^+-strain.

In addition to the incorporation of dNTP into plasmid DNA the deoxyribonucleoside triphosphates are mainly incorporated at 42° by toluene-treated cells of Dna B^--mutants into slow-sedimenting heterogeneous DNA material, which is hardly observed at 30°

Since in this mutant chromosomal DNA at 42° is rapidly degraded to DNA fragments [15], which may serve as templates for DNA polymerase I, the incorporation of dNTP into the small DNA fragments represents probably repair replication by DNA polymerase I.

Absence of ATP and addition of *p*-hydroxymercuribenzoate (6×10^{-5} M) to the reaction mixture again do not affect incorporation of dNTP into these DNA fragments (table 1). A drastic reduction of the incorporation of dNTP is observed with toluene-treated cells of CRT 2667 (Dna B^-, Pol A 1). Addition of pancreatic DNase to the reaction mixture, which has been shown to stimulate repair replication in toluenized bacteria [8] leads to an increase of incorporation of dNTP into the slow-sedimenting DNA material. In contrast, the colicinogenic DNA components, which sediment at 23 S and 17 S disappear completely in the presence of DNase (table 1). The results described above indicate that the incorporation

of dNTP into plasmid DNA parallels essentially the incorporation of dNTP into the small DNA fragments, which most likely represents repair activity of DNA polymerase I. We therefore conclude that the synthesis of Col E_1 DNA from deoxynucleoside triphosphates by toluenized cells of colicinogenic Dna B^--mutants is also performed by DNA polymerase I. The experiments described do not allow any conclusion whether the incorporation of dNTP into Col E_1 DNA is due to repair or to semiconservative replication. Similar conclusions have been reached by Kingsbury and Helinsky [17], who have demonstrated that Pol A 1 mutants with low DNA polymerase I activity ($< 2\%$) are not able to maintain Col E_1 DNA, while larger episomal elements are replicated in these mutants. Preliminary experiments have shown that dNTP are also incorporated into minicircular DNA [16] at the elevated temperature in toluenized cells of a Dna B^--type mutant of *E. coli* 15. In contrast incorporation of dNTP by suitable toluene-treated cells into Col V DNA and Hly DNA [18] (both are large transmissible extrachromosomal elements) are not observed at the elevated temperature.

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